The effect of stress on aspects of the immune system in the rainbow trout, with observations on the MCH neurones in the brain

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THE EFFECT OF STRESS ON ASPECTS OF THE IMMUNE SYSTEM IN THE RAINBOW TROUT, WITH OBSERVATIONS ON THE MCH NEURONES IN THE BRAIN.

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This thesis is dedicated with love to my Mum,

Smt. Parvati Khushalrao.

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Contents

CHAPTER 2.

GENERAL MATERIALS AND METHODS

CHAPTER 3.

FFECT OF DIFFERENT ACUTE AND CHRONIC STRESSES ON INNATE IMMUNE RESPONSE IN FISH.

CHAPTER 6.

EFFECT OF MILD AND INTENSE ACUTE STRESS, CORTICOSTEROID AND CATECHOLAMINES ON CIRCULATORY LEUKOCYTE NUMBERS IN TROUT.

ABBREVIATIONS

ACTH	Adrenocorticotropin Hormone
ADCC	Antibody-dependent cell cytotoxicity
ANS	Autonomic nervous system
ANOVA	One way analysis of variance
B-W	Fish transferred from black to white
cAMP	cyclic-adenosine monophosphate
CNS	Central Nervous System
SNS	Sympathetic Nervous System
Con.A	Concanavalin A
cpm	Counts per minute
CRF	Corticotropin-Releasing-Factor
CRP	C-Reactive Protein
Cyto.C	Cytochrome C.
DAB	Diamino-Benzaidine
DNFB	2,4, Dinitro-1-Flurobenzene
DTH	Delayed Type of Hypersensitivity
Fig.	Figure
FSH	Follicle-Stimulating-Hormone
G-CSF	Granulocyte-Colony-Stimulating-Factor
GCs	Glucocorticoids
GH	Growth hormone
GM-CSF	Granulocyte-Macrophage-Colony-Stimulating-
GVH	Graft-Versus-Host-Reaction
h	Hours
H_2O_2	Hydrogen Peroxide
HPA	Hypothalamo-Pituitary-Adrenal axis

HPI	Hypothalamo-Pituitary-Interrenal axis
I	Iodine
i.p.	Intraperitoneal
ICC	Immunocytochemistry
IFN-γ	Interferon-γ
IFN-α	Interferon-α
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-6	Interleukin-6
ir-	Immunoreactive
ISO	Isoproterenol
KLH	Key hole limpet haemocyanin
L-15	Leibovitz-15 medium
LC-1	Lipocortin-1
LH	Leuteinizing Hormone
LHA	Lateral hypothalamic area
LPS	Lipopolysaccharide
LVR	Lateral ventricular recess
M-CSF	Macrophage-Colony-Stimulating-Factor
MCH	Melanin-concentrating hormone
MCH-mRNA	Melanin-concentrating hormone mRNA
mg	miligrams
MHC	Major-Histocompatibility-Complex
MLR	Mixed-Lymphocyte-Reaction
NA	Nor-adrenaline
NEI	Neuropeptide Glutamine (E) Isoleucine (I)
ng/ml	Nanogram per mililitre

NK	Natural Killer cell
NLT	Nucleus lateralis tuberis
NLT pl	Nucleus lateralis tuberis pars lateralis
NLY pp	Nucleus lateralis tuberis pars posterioris
NPY	Neuropeptide Y
O^{2-}	Superoxide anion.
OT	Optic tectum
°C	degree Celsius
P	Probability
PAP	Peroxidase-anti-peroxidase
PBSG	Phosphate-Buffered Saline-gelatin.
PBST	Phosphate-buffered saline-Triton-X
PE	Phenylephrine
PEG	Polyethylene glycol
PGEs	Prostaglandins
PHA	Phytohaemagglutinin
PI	Phagocytic Index.
PMN	Polymorphonuclear cells
POMC	Proopiomelanocortin
PVO	Paraventricular organ
PRL	Prolactin
RBC(s)	Red Blood Cell (s)
sIg	Surface-Immunoglobulin
SLE	Systemic Lupus Erythromatosus
TNP-LPS	Trinitro-Phenoyl-Lipopolysacchrides
TSH	Thyroid Stimulating Hormone
U	Units
v/v	volume to volume ratio
VC	vulva of cerebellum
VIP	Vasoactive Intestinal Peptide

x g	Force due to gravity
%	Percentage
α-MSH	α-Melanocyte-Stimulating-Hormone
μCi	Microcurie
μg	Micrograms
^{3}H	Tritium
6-OHDA	6-Hydroxydopamine
3rd V	Third ventricle

Lists of tables and figures

List of tables

Lists of figures

SUMMARY

The work described in this thesis is mainly concerned with the effect of either brief (2 min), more prolonged (3 hr) or chronic stress on the innate immune system of trout. A brief saline injection or 3h noise plus confinement stress equally depressed the subsequent *in vitro* phagocytic activity of macrophages derived from the spleen and the pronephros. Surprisingly, repeated stress over 6 days tended to be less detrimental. When the recovery from a single stress was examined, phagocytic activity appeared to be enhanced one week after stress, but return to normal thereafter.

In vitro incubation of splenic and pronephric macrophages with cortisol failed to depress the phagocytic activity within 3h but depressed it within 6hr of incubation. By contrast, both the α- and ß- aminergic analogues, phenylephrine and isoprenaline depress the phagocytic activity within 3hr *in vitro*.

While the stress from saline injection or nor-adrenaline, depress both macrophage phagocytic activity and the number of circulating lymphocytes, cortisol injection prevented these stress-induced changes. Mechanisms by which this could occur are discussed.

This work also demonstrated some properties of a newly identified second group of Melanin-Concentrating-Hormone (MCH) neurones in trout brain and the response of these cells to changes in background colour.

SECTION I

FISH IMMUNOLOGY

CHAPTER 1

GENERAL INTRODUCTION

1.1 Need for Immunity.

Animals and plants, from the simplest to the most complex in oraganisation, are susceptible to harm from a vast range of infectious microbes and multicellular parasites. The maintenance of host integrity is critically dependent upon the removal of exogenous pathogens as well as dead cells and damaged connective tissue matrices. There is a need for a system which will eventually control pathogens from invading the organism's body.

Undoubtedly, the immune system play a major role in the attempt of a organism to resist pathogen invasion. The elements of this system are capable of employing different defence mechanisms, such as phagocytosis of invading pathogens and their elimination by antibodies and complement.

1.2 Immune system in mammals.

The immune system in animals evolved to protect them from infection by micro-organisms and larger parasites. In mammals, the immune system and its various components are well established. The main haemopoietic organs are bone marrow, lymph nodes and specialised lymphoid organs, such as the thymus, spleen, appendix and bursa of Fabricius in birds.

The bone marrow is the main source of all white blood cells in adult mammals (Alberts et al., 1994). The components of the immune system which first come in contact with invading pathogens, forming the first line of defence, include macrophages and neutrophils. Other components of the immune system which check infection consist of the extracellular chemical defence, including lysozyme which is secreted by macrophages and different proteins called 'acute phase proteins' and complement in the blood stream which increase during infection (Gordon, 1986; Dinarello 1992).

Other leukocytes of the mammalian immune system includes eosinophils, basophils and large granular lymphocytes known as NK-cells. Eosinophils and basophils are involved in parasitic killing and inflammation (Davey, 1989), whereas, natural killer (NK) cells kill tumour cells (Riley, 1981)

The second line of defence is provided by the different types of lymphocytes. These include B-cells which produce antibodies against pathogens and T-cells which are further sub-divided into T-helper, whose depletion can results in much increased susceptibility to infections, T-suppressor and T-cytotoxic cells (Maisel et al., 1990). These can be distinguished by their characteristic cell-surface proteins. For example, CD3 is found on all mature T-cells, CD4 on helper cells, CD8 on cytotoxic/suppressor and CD19 on B-cells (Bagasra et al., 1987; Maisel et al., 1990). T- cells are involved in cell-mediated immunity such as delayed type of hypersensitivity (DTH), contact sensitivity (Blecha et al., 1982) and graft-versus-host reaction (GVH) (Amat et al., 1993). In response to infection these cells produce a large number of cytokines by which they stimulate the activity of a wide range of cells, including macrophages, NK cells, and bone marrow

progenitor cells. When produced by macrophages in response to antigens, Interleukin-1 (IL-1) causes an elevation in blood cortisol and releases many pituitary hormones, such as adrenocorticotropic hormone (ACTH), growth hormone (GH), luteinizing hormone (LH), folliculo stimulating hormone (FSH), and prolactin (PRL) (Karnath and Mc Cann 1991).

1.3 Immune system in fish.

Fish lack bone marrow and the principal haemopoietic organs are the thymus, head kidney and the spleen (Ellis, 1977; Muiswinkel, 1992). The thymus provides the other organs with lymphocytes. The most important organ in the lymphoid system is the head-kidney (Rowley et al., 1988). However, unlike bone marrow, the kidney haemopoietic tissue contains many antibody-producing cells of immunity (Rijkers et al., 1980) and is a major site for formation of almost all cells of immunity (Lamers and Haas 1985). It also may serve as a primary lymphoid organ for B- cell maturation (Irwin and Kaattari 1980; Kaattari and Irwin 1985). The spleen contains fewer haemopoietic and lymphoid cells than the kidney. Immunostaining methods have revealed the presence of acid phosphatase and alkaline phosphatase (Ellsaesser et al., 1984; Clem, 1985; MacArthur and Fletcher 1985) demonstrating the phagocytic cells which form part of the innate immune system (Yoshida and Yamoto 1987; Razquin et al., 1990).

The immune cells of fish blood include about 85% of lymphocytes (Quentel and Obach 1992), depending upon the species, the balance being granulocytes, neutrophils, and thrombocytes (Rowley et al., 1988). However, macrophages are mostly present in

lymphoid tissues and are usually believed to be absent from the blood (Morgan et al., 1993) although, Sizemore et al., (1987) have said that they form 1% of leukocytes in the blood of catfish. Early studies of lymphocytes from elasmobranchs showed 60-80% of lymphocytes to be surface immunoglobulin (sIg) positive in the spleen, thymus and peripheral blood (Ellis and Parkhouse 1975; Lob and Clem 1982; Ellsaesser et al., 1985; Ellsaesser and Clem 1986). The immunoglobulin in fish is IgM-like (Ellsaesser et al., 1985) and located mainly in the blood and mucus and is produced against both particulate and soluble antigens (Ingram and Alexander 1977; 1980). Other components of the non-specific immune system are members of complement associated with lysis (Bower and Woo 1977) and / or opsonization (Miche et al., 1990); lysozyme and neutral proteases (Murray and Fletcher 1976; Alexander, 1985), and interferon-like molecules (Grondel and Harmsen 1985; Graham and Secombes 1990). The production of these components has been shown to increase in response to infection (de Kinkelin et al., 1982; Okamoto et al., 1983). Other substances which are increased in response to infection by antigenic challenge are: C-reactive protein (CRP) (Yen Watson 1968), transferrin (Weinberg, 1974), chitinase (Ingram, 1969), lysins (Grubb, 1949), agglutinins (Kantz and Hiest 1970), properdin (Lvkyaenko, 1969), and precipitin (Janssen and Meyers 1968).

T-cell mediated responses (Simson et al.,1980) and their co-operation with the B cell have been demonstrated in fish (Miller et al., 1986; 1987; Rowley et al., 1988;). The presence of T-cell subtypes such as cytotoxic T-cells (Kajita et al., 1992), helper and suppressor cells (Lopez et al., 1974; Avtalion, et al., 1980) and their responses such as mixed lymphocyte reaction, (Kastrup et al., 1988) and histocompatibility (manifested by

the ability to reject allograft) have been described (Sailendri et al., 1973; Miller et al., 1986; Kastrup et al., 1988). The existence of lymphocyte heterogeneity (Etlinger et al., 1976; Sizemore et al., 1987), immunological memory (Paterson and Fryer 1974; Tatner, 1986) and immunological tolerence (Manning et al., 1982) has also been described in fish. Moreover, the production of a macrophage activating factor and cellular requirement for lymphokine secretion by leucocytes have been demonstrated previously (Graham and Secombes 1990). It is well known now that fish can secrete interferon (IFN) molecules in response to viral infection of fibroblast or epithelial cell line *in vitro* (Okamoto et al., 1983) and *in vivo* (de Klinkelin et al., 1982). In addition, antigen presentation along with major-histocompatibility (MHC) molecules have been well established in fish but our knowledge of the molecular structure of the MHC molecules is still rudimentary (Vallejo et al., 1992).

1.4 Comparison of fish with mammals.

The immune system of fish has not been as extensively studied as that of mammals but the two appear to share a number of structural and functional characteristics important in the humoral, cell mediated and non-specific aspects of the immune responses (Ellis, 1977). Many aspects of immune mechanism in fish are similar to those of mammals.

The immune system of fish, similar to that of mammals, include T- and B- cell lymphocytes and non-specific cytotoxicity (Kajita et al., 1992). The use of immunoassays that are normally used in mammals show that, like mammals, fish macrophages upon stimulation produce hydrogen peroxide (H_2O_2) (Stave et al., 1983; Angelidis et al.,

1987) and superoxides (O^{-2}) (Bayne and Flory 1991) and their lysosomes contain acid phosphatase and alkaline phosphatase. Enzymes are present in the plasma membrane of macrophages and these cells perform phagocytosis (Enane et al., 1993). Moreover, histochemical and ultrastructural studies revealed that fish macrophages and neutrophils closely resemble their mammalian counterparts (Mac Arthur and Fletcher 1985) but little is known about their functional aspects, such as factors affecting their phagocytic responsiveness etc. However,, significant differences also exists. For example, the proposed function of the kidney as a source of stem cell (Ellis, 1977), its haemopoietic capacity, ultrastructure (Zapata et al., 1979) and the histocompatibility features of its stromal cells resembles mammalian kidney (Castello et al., 1987). But significant differences also exists in the defence mechanism of fish species.

The most significant difference between mammals and fish is the presence of only IgM in fish sera (Ingram and Alexander 1977; Dorson 1981) and the failure in fish to switch to the production of non-IgM classes of antibodies during the secondary immune response (Wilson and Warr 1992). Fish IgM resembles mammalian IgM (Litman, 1975) in its characteristic features in terms of heavy chain mass, interchain disulphide bonding, amino acids, carbohydrate content and confirmation (Litman, 1975). However, the difference between the maximum titre of the primary and the secondary immune response was found to be less in carp (Avtalion, 1969; Ambrosius and Frenzel 1972), and gold fish (Trump and Hilderman 1970) than seen in mammals. Also, after the second injection of Lipopolysacchrides (LPS), the maximum titre was reached only after 56-63 days, i.e. much more slowly than in mammals, and was not significant after third injections

compared to control (Ingram and Alexander 1980), showing the response had not risen progressively with successive challenge, as one find in mammals.

As indicated earlier lymphocyte heterogeneity (Ellis, 1977), mixed lymphocyte reaction (MLR) (Kastrup et al., 1988), immunological memory (Paterson and Fryer 1974) and immunological tolerance (Manning et al., 1982) have been considered to occur in fish as in mammals. Immediate hypersensitivity in fish also occurs (Ellis, 1982). In mammals, this reaction is elicited by IgE binding to mast cells but in fish, IgE does not exist and typical mast cells are absent (Ellis 1981a). Instead, eosinophilic granular cells play a role in hypersensitivity in salmonids (Baldo and Fletcher 1975). These authors have reported localised degranulation of this cell in plaice undergoing a skin hypersensitivity response.

Regarding the complement system in fish, Ellis (1977) has shown similarity of fish complement components with their mammalian counterparts except for the presence of only 6 components in fish, compared with nine in mammals of which three showed compatibility with three of the mammalian components. The role of complement in lytic activity is similar to mammals (Nonaka et al., 1981). Nevertheless, fish complement is needed to elicit a plaque reaction after immunising fish with sheep red blood cells (RBCs). Presence of cytokines such as IL-1, IFN and their action is also similar to those of mammals (Graham and Secombes 1990).

1.5 Effect of stress on mammalian immune system.

The effect of stress upon the immune system are only partially understood in mammals.

It is generally accepted that physical and psychological stress can impair immune function and decrease disease resistance (Mojan and Collector 1977; Riley, 1981; Landslaugher et al., 1983). The influences of stress on immunity are mediated by neuroendocrine responses (Stein et al., 1985), and a neuroendocrine-immune system regulatory axis is proposed. Stress in mammals causes a decrease in lymphocyte proliferation (Keller et al., 1983; Lysle et al., 1987; Jain et al., 1991), reduces phagocytosis (Garabal et al., 1993) and natural killer cell activity (Pollock et al., 1987; Irwin and Haugher 1988; Irwin et al., 1988; Cunnick et al. 1988; Irwin et al., 1991). It also affects the B-cell responses such as a decrease in antibody production (Wan et al., 1993; Coe et al., 1993; Amat et al., 1993) and T-cell mediated responses such as graft-versus-host reaction (Pollock et al., 1987; Amat et al., 1993). It decreases delayed hypersensitivity (Okimura et al., 1986), increases the rate of tumour spreading (Pollock et al;, 1987) and lowers the number of lymphocytes in the circulation (Murray et al., 1992; Dhabhar et al., 1994).

One of the effects of stress is the activation of the Hypothalamo-Pituitary-Adrenal axis (HPA) in mammals. The resultant glucocorticoids have many wide ranging actions, affecting many aspects of bodily functions including metabolism, inflammation and immunity. Thus, glucocorticoids (GCs) are anti-inflammatory and block the production and action of several lymphokines such as interleukin-2 (IL-2) and Interferon-gamma (IFN-γ) and prostaglandins (PGEs) (Spangelo et al., 1991; Boumpas et al., 1991; Paliongianni et al., 1993). In addition, they can block IL-1 production by macrophages and also inhibit the induction of surface molecules necessary for antigen presentation (Synder and Unane 1982).

In mammals, other effects of glucocorticoids (GCs) on immune responses are to reduce the number of circulating monocytes (Thompson and van Furth 1972; Parillo and Fauci 1978; Boumpas et al., 1991) or cause them to be sequestered in the bone marrow (Cohn, 1968). They also block phospholipase activity which is responsible for prostaglandin and leukotriene production, and block the production of certain proteases involved in inflammation (Besodovsky et al., 1986). They also depress aspects of macrophage metabolism which includes cell growth, protein synthesis and net glucose uptake and lactate production (Norton and Munk 1980; Costa-Rosa et al., 1992), their chemotactic response (Norris et al., 1977) and phagocytosis (Costa Rosa et al., 1992). finally, corticosteroid receptors have been demonstrated in mammalian leukocytes (Plaut, 1987).

In mammals, repeated stress for several days (chronic stress) continuously challenges the animal and under these circumstances exhibits habituation or adaptation of neuroendocrine and immune responses (Mojan and Collector 1977; Spencer and Mac Evans 1990). During chronic stress plasma levels of corticosterone, ACTH, corticotropin-releasing-factor (CRF), and other hormones/neurotransmitters remain elevated for up to 2-4 weeks and subsequently fall back towards control level (Vermikos et al., 1982). Repeated footshock (Kant et al., 1985), restraint (Hashimoto et al., 1985), sound (Mojan and Collector 1977), and immobilization for many hours (Irwin and Haugher 1988) are the modes of delivering chronic stress in mammals.

On the other hand, stress or stress-induced release of glucocorticoids or catecholamines are not always immunosuppressive. Some stressors in mammals, such as exercise (Fehr

et al., 1988; 1989; Ortega et al., 1993), hot or cold shock (Aastrad et al., 1991) and sound (Mojan and Collector 1977) and physical stimulation (Fujiwara and Orita 1987) have actually been shown to increase cellular (Targan et al., 1981; Wood et al., 1993), and humoral (Fehr et al., 1988) immune responses. Glucocorticoids administered to macrophages *in vitro* enhances phagocytosis under stress condition (Kay and Czop 1992), while *in vivo* administration of epinephrine (Tonnesson et al., 1984; Fujiwara and Orita 1987) enhance the number of plaque-forming cells (PFC).

1.6 Sympathetic nervous system and immune system in mammals.

The role of corticosteroid-independent effects of stress on immunity are less well documented. It is known, though, that a classical stress response involves activation of the sympathetic and parasympathetic nervous system. Growing evidence suggests that this system plays an important role in modulating functional parameters of the immune system. It has been shown that lymphoid tissues in mammals are innervated with sympathetic nerve fibres (Felten et al., 1987) and that immune cells possess adrenoreceptors of high affinity and specificity (Williams et al., 1979; Sanders and Munson 1985). The activation of this system results in the release of the catecholamines, adrenaline and nor-adrenaline. Administration of adrenaline or nor-adrenaline has been shown to affect many immune parameters. Thus, stress-induced release of catecholamines, or their *in vitro* application, have been shown to inhibit activation of macrophages to a tumoricidal activity and anti-viral state (Koff and Dunegan 1985; 1986), to depress blastogenic activity of blood lymphocytes (Crary et al., 1983), increase plaque-forming-cells (PFCs) (Fujiwara and Orita 1987) and decrease adherence

of polymorphonuclear cells (PMN) to substrates (Sheng et al., 1983) and reduce NK cell activity (Tonnessen et al., 1984).

In *in vitro* studies, catecholamine analogues such as isoproterenol have been shown to inhibit O^{-2}, H_2O_2 production (Nielson 1987) and depress mitogen-induced lymphocyte proliferation (Murray et al., 1992; 1993). Pre-treatment with propranolol prevented stress-induced immune suppression while isoproterenol caused a decrease of immune parameters, suggesting that catecholamines exert their effects through β-adrenergic receptors. Chemical sympathectomy with the neurotoxin, 6-hydroxydopamine (6-OHDA) increases cellularity of lymph nodes, spleen and bone marrow (Madden et al., 1994), whereas administration of desipramine (an antagonist of 6-OHDA) prevented this increase in cellularity, suggesting that the sympathetic nerves exert a depressive effect on the immune system.

One of the effects of catecholamines on the immune system is to cause lymphocytosis (Crary et al., 1983; Maisel et al., 1990; Murray et al., 1992; 1993). Stress-induced release of catecholamines caused an increase of β-adrenoreceptors on some lymphocytes and caused their release into the circulation (Maisel et al., 1990). Thus it would appear that the sympathetic nervous system plays an important role in the migration of immunoregulatory cells from lymphoid organs into the circulation or vice versa. Whether catecholamines and cortisol have effects on the homing receptors of lymphocytes i.e. the receptors which recognise binding sites in the lymphoid glands, thereby causing them to lose their adherence and increase their numbers in the

circulation, is not known currently. The observed changes in immune responses may be due to a change in lymphocyte subsets.

Under stressful situations, one sees the release of many neuropeptides, one of them being CRF. CRF acts as an endogenous neurotransmitter in the brain and primarily regulates pro-opiomelanocortin (POMC)-derived peptides from the pituitary gland (Levin et al., 1989). It also initiates other biological responses within the brain that are observed in response to stress, suggesting that endogenous CRF functions as a stress neurotransmitter in the central-nervous-system (CNS) to produce effects on behaviour (Sutton et al., 1982) and activates the autonomic nervous system (ANS) enhancing plasma catecholamine levels (Brown et al., 1982). Its production also in lymphoid tissues (Taylor and Fishman 1988) suggests that it may have additional effects on the immune system. Not only the presence of CRF (Hollander et al., 1983) but also CRF mRNA (Thompson et al., 1987) and CRF binding sites on lymphoid tissues has been demonstrated in mammals (Dave et al., 1985). It stimulates the production of ACTH and β-endorphin by leukocytes (Smith et al., 1986) and these products might also mediate CRF action on O^{-2} generation (Sharp et al., 1985).

The administration of CRF to rats and mice causes a decrease in splenic (Brown et al., 1982; Irwin and Haugher 1988; Jain et al., 1991; Irwin et al., 1992), peritoneal and blood NK cell activity (Strausbaugh and Irwin 1992). Other work shows a direct effect of CRF on NK *in vitro* (Audhya et al., 1991). CRF also reduces the magnitude of the IgG response to a specific T-cell dependent antigen (Irwin, 1993). These effects are mediated through CRF receptors on rat splenocytes (Dave et al., 1985). Chemical

sympathectomy or non-selective β-antagonists, antagonized the CRF-induced suppression of NK cell activity. It is now believed that apart its role in the brain and autonomic nervous system activation, CRF is also produced in the spleen where it directly influences the immune system.

In the brain, CRF is produced not only in response to stress, but also in response to infections, which can induce IL-1 production by astrocytes and microglia (Bredar et al., 1988). It is this centrally-produced IL-1 which stimulates CRF production and release. Thus cytokines will depress NK cell function indirectly, by an effect on CRF production (Saperstein et al., 1992).

1.7 Effect of stress on fish immune system.

The effect of stress on the fish immune system is under intensive investigation and many potential responses have not yet been investigated. Stress in fish can result in outbreak of infectious disease (Walter and Plumb 1980; Fries, 1986; Maule et al., 1989). The response of the immune system to stress includes the reduction of lymphocyte proliferation (Fries, 1986; Maule et al., 1987; 1989), reduction of antibody production (Ellsaesser and Clem 1986; Tripp et al., 1987; Maule et al., 1989) and depressed phagocytosis (Narnaware et al., 1994; Pulsford et al., 1994). Stress also induces cellular changes in the haemopoietic tissues of the spleen and kidney (Peters and Schwarzer 1987), reduces inflammation (MacArthur et al., 1984), hydrogen peroxide and superoxide production by phagocytes (Anglidis et al., 1987; Whiskovsky et al., 1987), production of lysozymes (Angelidis et al., 1987; Mock and Peters 1990;), complement

formation and lowers the number of lymphocytes in the circulation (Pickford et al., 1971 a, b; Pickering et al., 1982; Ellsaesser and Clem 1986; Pickering et al., 1987; Wiik et al., 1989; Pickering and Pottinger 1992; Pulsford et al., 1994;).

However, as compared to mammals, the proven effects of glucocorticoids or cortisol on fish immune system are limited and are under investigation. Stress-induced corticosteroids are known to suppress the immune system in fish. An increase in plasma cortisol, either due to an acute stress or to exogenous cortisol treatment, results in a marked lymphocytopenia (Pickering et al., 1982; Pickering et al., 1984), while the remaining circulating leukocytes show decreased mitogenic and antibody responses *in vitro*, and the fish shows increased susceptibility to disease (Pickering and Duston 1983; Pickering 1984; Grimm, 1985; Ellsaesser and Clem 1986; Maule et al., 1989; Wiik et al., 1989). Cortisol applied at physiological levels *in vitro* directly suppresses antibody production (Anderson et al., 1982; Tripp et al., 1987). Cortisol probably operates by down-regulating the effect of certain cytokines (Kaattari and Tripp 1987; Tripp et al., 1987) via receptor mediated processes.

In fish, as in mammals, chronic stress influences the immune system but it is not yet known whether habituation occurs. Elevation in temperature and pollution are examples of natural chronic stressors. Overcrowding (Pickering and Stewart 1984), confinement (Pottinger and Pickering 1992), or forced swimming (Woodward and Smith 1988) for several days have been shown to affect growth, to increase mortality and decrease the number of lymphocytes in the circulation (Barton et al., 1987; Pottinger and Pickering

1987; Pickering and Pottinger 1992). However, the effects of these treatments on the innate immune response have not been investigated.

1.8 Sympathetic nervous system and immune system in fish

In fish, lymphoid organs such the spleen contain a rich adrenergic innervation (Nilsson and Grove 1974; Flory, 1988) and lesioning of adrenergic nerve terminals with 6-OHDA results in an enhanced number of antibody-secreting cells in immunized trout (Flory, 1989). Subsequent studies on rainbow trout (*Oncorhynchus mykiss*) have established that adrenergic and cholinergic agents can influence directly the function of leukocytes *in vitro* (Flory, 1990) Thus, β-adrenergic receptor agonists reduced, while α-adrenergic and cholinergic agonists increased, the number of the cells secreting antibody in response to trinitrophenyl-lipopolysacchrides (TNP-LPS) (Flory, 1989) in splenic leukocytes. They also altered the chemiluminiscence response in pronephric leukocytes (Flory and Bayne 1991), but whether cytotoxicity was enhanced or depressed depended upon the methods of their measurements (Bayne and Levy 1991a, b). The enhancement due to phenylephrine was blocked by the α_2-antagonist, yohimbine and that due to carbachol was blocked by atropine suggesting that, as in mammals, both the sympathetic and parasympathetic nervous systems regulate the immune system in fish (Bayne and Levy 1991) (see figure 1.1). However, catecholamine-induced changes in fish leukocyte distribution deserve further investigation.

Some evidence suggests that this stress response in fish is modulated not only by the corticosteroids but also by opioids (Bird et al., 1987; Mukherjee et al., 1987) and sex steroids (Schreck et al., 1989).

1.9 Effects of opioids on mammalian and fish immune system.

The significance of endogenous opioids on the mammalian immune system remains a matter of speculation, although numerous stimulatory and suppressive effects of opioids on immunity have been reported (Sibinga and Goldstein 1988). For example, β-endorphin enhances leukocyte mitogenic responses (Gilman et al., 1982) and auguments NK cell activity (Matthews et al., 1983), although stress-induced elevation of opioids have been shown to reduce NK cell activity (Shavit et al., 1984).

In fish, β-endorphin is known to be released from corticotrophs of the pituitary gland in response to stress (Carragher and Sumpter 1990), but its effect on the immune system in not yet clear.

1.10 Effects of other hormones and neurotransmitters on mammalian and fish immune system.

Other neuropeptides may modulate immune function by a direct effect on leukocytes. For instance, substance P enhances mitogen responses (Pyan et al., 1983), whereas vasoactive intestinal peptide (VIP) inhibits them (Ottaway and Greenberg 1984). VIP also inhibits NK cell function (Rola-Pleszczynski et al., 1985) or potentiate

the inhibitory effect of nor-adrenaline (Koff and Dunegan 1985). Neurotensin increases the cytotoxic capability of macrophages activated with IFN-γ (Koff and Dunegan 1985).

Hormones have important roles in mammalian immunoregulation. In particular, growth hormone (somatostatin) which is actually synthesized by leucocytes, is known to enhance activities of the thymus gland, lymphoid cells, phagocytic and stem cells (Kelley, 1989).

It has not yet been determined whether somatotropin has similar effects in fish. It has been thought that stress might suppress circulating somatotropin levels in the short-term, but chronic stress may promote an increase in plasma somatotropin (Pickering, 1990). A number of immunosuppressive effects has been shown to be reversed also by prolactin in mammals (Nagy et al., 1983) and in fish (Kajita et al., 1992).

1.11 Neuroendocrine regulation of mammalian immune system.

Neuro-immuno-endocrinology is becoming an increasingly important discipline of immunology. Several hormone and neuropeptides which are normally produced and released by nervous and endocrine tissues are now known to be produced by immune cells. These include ACTH (Blalock and Smith 1980), endorphin and enkephalin (Smith and Blalock 1981; Lolait et al., 1986), thyroid-stimulating-hormone (TSH) (Smith et al., 1983; Kruger 1989), GH and PRL (Goetzl et al., 1985; Hiestand et al., 1986) and vasoactive-intestinal-peptide (VIP) (Cutz et al., 1978). Most of these hormones and neuropeptides have been detected in lymphoid organs (Felten et al., 1985; Lorton et al., 1991). Moreover, POMC gene expression has been obsereved in peripheral mononuclear

cells (Buzzetti et al., 1989) and POMC effects are mediated through specific receptors present on lymphocytes (Bar-shavit 1982; Danek et al., 1983). Some of these neuropeptides are produced by the nerves that innervate lymphoid organs. Thus, immunoregulatory neuropeptides may arrive at the immune cells from two sources- they may be produced locally or be released from innervating neurones.

Knowledge of the bi-directional communication of these systems came from the observation that antigenic challenge can cause an increase in the firing rate of neurones in the hypothalamus (Besodovsky et al., 1977), and can cause the immune cells to produce small quantities of ACTH, β-endorphin, and certain hormones such as TSH (Blalock et al., 1985). Immunization with sheep-red-blood cells (sRBC) changes the hypothalamic and brain-stem content of nor-adrenaline (Besodovsky et al., 1979; Del Rey et al. 1982) and removal of the splenic nerve increased antibody production in the rat spleen (Besodovsky et al., 1979). Immune cell products, such as IL-1 are produced by activated glial cells in the brain, when administered in brain they increase slow wave sleep (Kruger et al., 1984). These products activate the HPA axis through CRF, providing the important link between immune and endocrine system (Rivier and Vale 1989), implying that immune cells have the ability to modulate the neuroendocrine system (Besodovsky et al, 1985).

1.12 Neuroendocrine regulation of fish immune system.

A similar neuro-immuno-endocrine relationship almost certainly occurs also in fish. Evidence provided by Levy and Bayne (1989; 1990); Bayne and Levy (1991 a,b;) and

Flory and Bayne (1988) have demonstrated that products of the sympathetic and parasympathetic nervous system modulate the immune function through receptors present on leukocytes (Flory, 1990). ACTH and thyroid hormone have been found to decrease the number of circulating white blood cells, whereas sex hormones and prolactin did not have any effect on the cell number (Slicher, 1961). However, the action of these and other neuropeptides and hormones have been studied mainly on the humoral immune system but their production by immune cells and their action on the neuroendocrine system has not yet studied in fish nor has their effect on the innate immune system of fish has studied.

1.13 Macrophages and their importance in mammalian immune system.

Phagocytosis, the cellular ingestion of particulate matter, is the most widely distributed defence mechanism occurring virtually in all animal phyla. Macrophages participate in the defence of an organism in a number of ways: they remove debris tissue during involution, they destroy pathogenic organism by phagocytosis (Davey, 1989). They exhibit a number of cytotoxic mechanisms against infection by producing H_2O_2 (Longering and Schwacha 1991), superoxide anion (Koshida and Kotake 1994) and nitric oxide (Liew et al., 1990), and play an important role in host defence against neoplasia (Nathan et al., 1980). Their lysosomes contains a variety of neutral proteinase, acid hydrolyses and plasminogen activator which help rapid intracellular degradation of ingested substances (Werb, 19768 Nathan, 1980). They also serve as antigen-presenting cells, so that mononuclear cell interaction leads to the recruitment of B cells, synthesis of specific antibody and the ability to express humoral immunity (Davis, 1989).

When activated, macrophages produce cytokines such as IL-1, interleukin-6 (IL-6) and tumor-necrosis-factor (TNF). IL-1 is a mediator of immune and inflammatory responses. It exhibits a diverse range of biological activities. These include augumentation of T-lymphocyte proliferation and thus, ultimately, antibody production (Howard et al., 1983; Limpskey 1983) and also fibroblast proliferation and prostaglandins production (Schmidt, 1982; Dinarello, 1984). IL-1 can stimulate the production of granulocyte-macrophage-colony-stimulating-factor (GM-CSF), macrophage-colony-stimulating-factor (M-CSF) and TNF-α by endothelial cells and fibroblast and can stimulate the release of colony-stimulating-factor (CSF) by haemopoietic progenitors. It activates the expression of granulocytes stimulating factor (G-CSF) and and causes the secretion of acute phase proteins by the liver (Dinarello, 1986). As described previously, IL-1 also modulates the release of CRF with its consequent effects on immune function.

The peripheral administration of IL-1 causes hypoglycemia by increasing insulin secretion (Del Rey and Besodovsky 1989) by unknown routes. Incubation of pituitary tissue with IL-1 causes the release of a number of pituitary hormones such as ACTH, LH, GH and, inhibits PRL release (Bernton et al., 1987) and causes the synthesis/release of IL-6 (Spangelo et al, 1991).

In mammals, the sympathetic nervous system have been shown to modulate the macrophage functions as well as the lymphocytes functions discussed earlier. Thus, sympathectomy enhances the macrophage function measured by increased TNF secretion, while isoproterenol depresses it (ChelmickaSchorr et al., 1992), thus providing

an important link between the CNS and the innate immune system. In mammals, studies have demonstrated the effect of stress-induced neuropeptides modulating macrophage functions. For instance, VIP (Litwin et al., 1992), TSH (Costa-Rosa et al., 1992), morphine (Rojavin et al., 1993), epinephrine, insulin and somatostatin (Bermudez et al., 1992) all decrease macrophage phagocytosis and chemotaxis (Litwin et al., 1992) by increasing cAMP. Other endocrine molecules such as bombesin, gastrin-releasing-peptide (GRP), neuromedin C and N (Bar-Shavit et al., 1982; De La Fuente et al., 1990; 1993) and thyrotropin-releasing-hormone (TRH) (Koshida and Kotake 1993) stimulate murine macrophage processes: chemotaxis, phagocytosis, superoxide production and adherence to substrate. Although the major focus of these hormones/neuropeptides and neurotransmitters is under investigation on innate and humoral immune system in mammals, there is a paucity of information concerning the regulation of these systems in fish, the whole problem needs further investigation.

1.14 Importance of macrophages in fish immune system.

In fish, although, the immune system is sophisticated and complex, innate immunity, including the activity of the macrophages, probably provides a particularly important initial defence against pathogens. The wide distribution of phagocytic cells in all tissues and lympoid organs enable them to keep a check upon infection (Ellis et al., 1976; Ellis, 1977; Braun-Nesje et al., 1981). There is evidence to suggest that macrophages in fish perform roles similar to mammalian macrophages. MacArthur and Fletcher (1985) suggested that there is a difference between the teleost and mammalian immune system in that macrophages seem to be of greater important in fish. Antibody production is slow

at the normal environmental temperature of cool-water fish (Avtalion et al., 1973; Cottrell, 1977; O'Neill, 1980). Many authors have studied the effect of heavy metals such as cadmium, copper (Mushike et al., 1985; Thuvander et al., 1989) on macrophage activity and drugs like tetracycline (Whiskovsky et al., 1987) have also been shown to affect the macrophage activity of fish.

1.15 Aims of the present study.

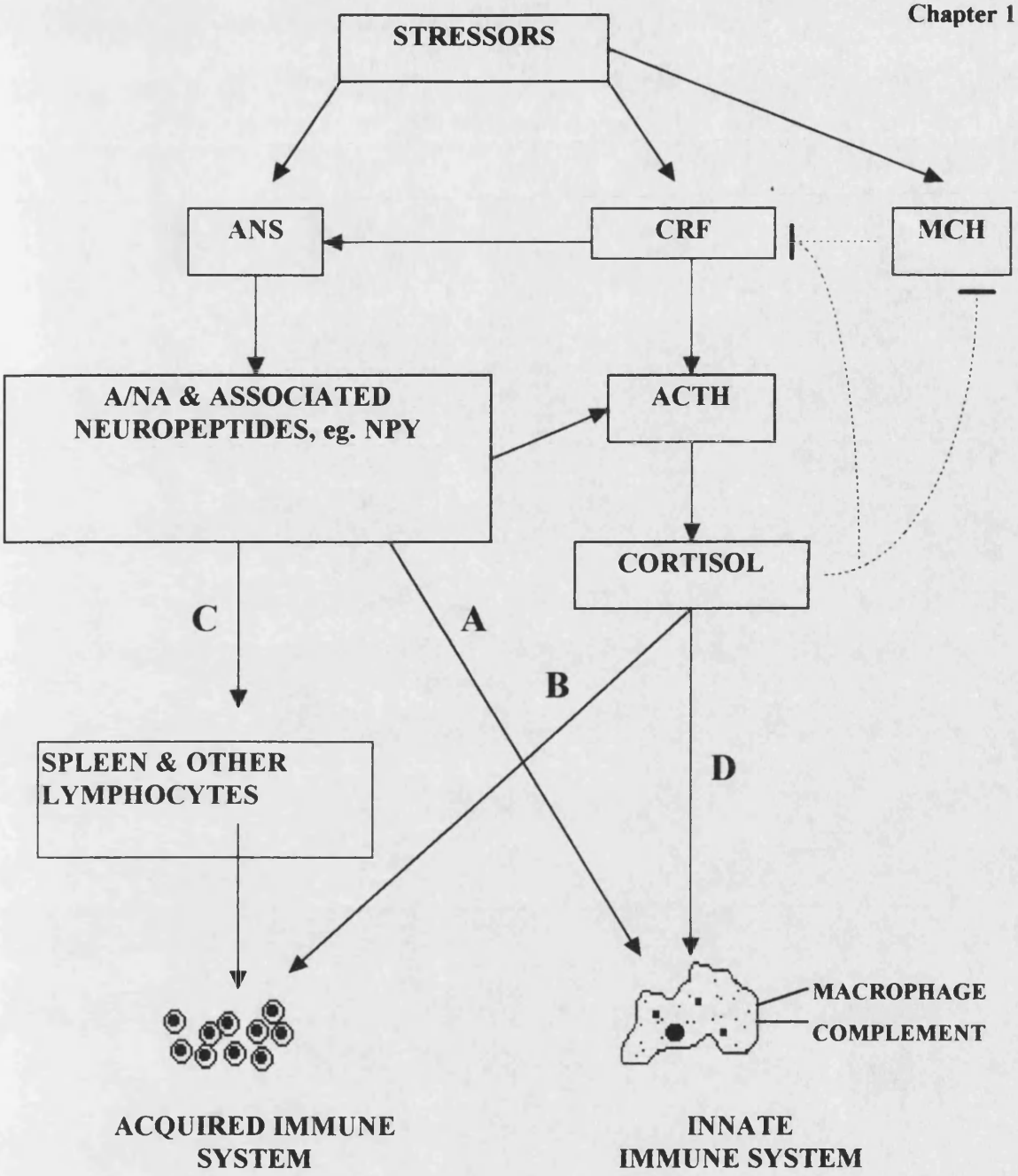
It is not yet known that how long a stress has to be applied for in order to exert a detectable effect on the innate immune system. Most work on fish has examined the effect of prolonged stress (i.e. pollution etc). The influence of brief stress has been examined only with respect to the acquired immune system i.e. the responsiveness of the lymphocytes. The aim of the present work, has been to apply stresses for only two minutes up to 3hr, and examined the effect of this on one aspects of the immune system viz. the phagocytes.

Another aim has been to try and determine, by *in vitro* as well as *in vivo* approaches, which components of the stress response (adrenaline/nor-adrenaline or cortisol) is responsible for any change in immune function.

Other studies from this laboratory have suggested that the stress response and the degree of the activation of the HPI axis is modulated by the melanotropic neuropeptide, Melanin-Concentrating-Hormone (MCH), which is preferentially released in fish adapted to a white background. Fish adapted to white tank appear to

have less active HPI axes, as judged by their release of cortisol in reponse to stress. It is believed that MCH may depress CRF release (Green et al., 1991) and hence damp down HPI activity. Additionally, cortisol will depress the release of MCH (Green and Baker 1991) and it may be through the feedback response that fish reared in white tanks, with reduced cortisol feedback on the hypothalamus, seem to be accumulate more CRF in their hypothalamus (Green et al., 1991).

Moreover, a second melanotropin, α-Melanocyte-Stimulating-Hormone (α-MSH) has been shown to modulate certain aspects of the immune system eg. response to interleukin-1 (IL-1) and this peptide too, is influenced by tank colour in trout and its effects on melanophores is antagonized by MCH. Thus, another line of investigation was to see whether tank colour, by influencing MCH and the HPI axis, would affect aspects of the immune system- in this case, the level of circulatory lymphocytes.



A) Investigated *in vitro* on phagocytic chemiluminiscence (Bayne and Levy 1991a,b).

B) Investiged *in vitro* on antibody production (Maule et al., 1987; Anderson et al. 1986).

C) Investigated *in vitro* on mitogenic response of lymphocytes (Flory, 1988; Flory and Bayne 1990).

D) Investigated *in vivo* effect on inflammatary response and *in vitro* on complement (Mac Arthur et al., 1985: Kaige et al., 1990).

Figure 1.1. The effect of stress and stress-induced hormones and neurotransmitters on aspects of the immune system. For abbreviations, see page i-ii.

CHAPTER 2

MATERIALS AND METHODS

This chapter deals with materials and methods which are common to all chapters except chapter 8 on immunocytochemistry of MCH. Treatments applicable to specific sections of the work are described under the appropriate chapter.

2.1 Fish.

Rainbow trout, *Oncorhynchus mykiss* of approximately 200-325g were obtained from Alderely Trout Farm, Wotton-Under-Edge, Gloucestershire or reared under aquarium conditions (Bath reared). The fish were housed in black or white 250 litre capacity fibreglass tanks in continuous flowing water (100 ml/min.). Fish were fed on a daily diet of trout pellets, under a lighting regime of 17h light:7h dark, at room temperature of 11 $\pm$ 0.5 ^{o}C. All fish were adapted to aquarium conditions for at least 2 weeks unless otherwise stated.

2.2 Collection of blood and storage of plasma.

Fish were anaesthetised in phenoxyethanol (diluted 1:1667 v/v). They were bled from the caudal vein into a ice cold 4 ml polypropylene tubes containing 50 µl 6% EDTA disodium salt (w/v) as anticoagulant. The blood samples were then centrifuged at 3000g for 15 min, and the plasma was stored at -20 ^{o}C in the appropriate aliquots.

2.3 Preparation of pronephros and spleen suspension.

The pronephros and spleen were dissected out. Cell suspensions of each were obtained by pushing the tissue with a steel spatula through nylon mesh into 4 ml Leibovitz-15 medium (Sigma), supplemented with 0.1% fetal calf serum and 4U/ml heparin (Sodium salt grade, II). The pronephros cell suspension was diluted 1:4 before use in the phagocytosis assay because of the relatively large numbers of macrophages present in this tissue.

2.4 Yeast cell preparation.

Heat-killed yeast cells were prepared by mixing 0.1g of commercial Baker's yeast (*Saccharomyces cerevisiae*) in 20 ml 0.15M Phosphate buffered saline, PBS (pH 7.8) and heating the suspension in a water bath at 80 ^{o}C for 15 min. The cells were washed with centrifugation 3-4 times in PBS and finally suspended in L-15 medium supplemented with 4% fetal calf serum (FCS) to give a concentration of appropriately 2.4 x 10^{8} cells/ml.

2.5 Phagocytic assay.

The method employed to measure macrophage phagocytosis was a microscopic counting technique based on the method described by Weeks and Kavas (1979). Slides, prewashed in soapy water and thoroughly rinse in distilled water were, flooded with 2 ml pronephros or spleen cell suspension in L-15 medium plus 0.1% FCS and the cells were allowed to settle for 90 min at room temperature.. Non-adherent cells were

washed off with 0.15M PBS, pH 7.8, and the slide was flooded with 2 ml heat-killed yeast suspension. Phagocytosis was allowed to proceed for 90 minutes at room temperature of about 20 °C.

Slides were then rinsed in 0.15 M PBS, fixed in methanol for 10 min, followed by staining with May/Grunwald stain (Sigma) for 2.5 mins. Slides were then soaked in 0.02M PBS (pH 7.8) for 5 min and transferred to Giemsa stain (Sigma; diluted with distilled water at 1:20 dilution) for 5 min, followed by a rinse in deionised water and air dried. The cells were examined at x100 under oil immersion.

Two counting methods were employed to quantify macrophage phagocytosis. Firstly, the average number of yeast cells engulfed per macrophage was determined by inspecting 200 macrophages from each fish. This is termed the Phagocytic Index (P.I.). From these counts was also determined the percentage of macrophages that engulfed one or more yeast cells. This was termed the % phagocytosis.

2.6 Lymphocyte counts.

The change in plasma lymphocyte numbers that occurred in response to different experimental conditions was evaluated after staining blood with Rees-Ecker stain (Klontz and Smith, 1968), prepared as follows:

3.8 g	disodium hydrogen citrate (BDH)
0.1 g	Brilliant cresyl blue (BDH)
0.2 ml	formaldehyde 40%
100 ml	distilled water

The solution was mixed thoroughly and filtered. It was stable at room temperature for at least 3 months. Immediately before use, Rees-Ecker stain was diluted 1:3 with 3.8% disodium citrate solution .

To count the lymphocytes, 50 µl of blood was diluted in 950 µl Rees-Ecker (1:20 dilution) and mixed for 10 min on a tumbler. Lymphocytes were counted in a haemocytometer (Neubauer) under a microscope at x40 magnification and expressed as thousands of cells per µl of blood by using following formula:

$$\text{Thousands cells/µl} = \frac{\text{Number of leucocytes in mm squares x dilution x10}}{\text{Number of 1mm squares counted}}$$

2.7 Radioimmunoassays.

Cortisol

Cortisol was measured by the method used by Rance and Baker (1981) and a brief description is given below:

a) Sample preparation.

Measurement of cortisol in plasma requires ethanol to precipitate the carrier proteins and liberate the steroid before assay. This was done in a following way:

- 100 µl plasma was used per tube in duplicate.

- 500 µl EtOH was added to each tube which was then vortexed.

- A further 500 µl ethanol was added.

- Tube contents were dried under vacuum.

- Contents were resuspended in 200 µl PBSG containing ^{3}H cortisol and cortisol antibody for RIA.

b) Buffer preparation.

The buffer used through out was phosphate buffered saline 0.05M pH 7.4, containing 0.9 % NaCl and 0.1% gelatin (PBSG buffer).

c) Cortisol standard.

A stock buffer containing 1.6 mg synthetic cortisol (Hydrocortisone, Sigma) was dissolved in 10 ml ethanol (BDH). 25 µl of this stock was resuspended in 25 ml of ethanol and subsequently diluted to give 1600 pg/10 µl. It was then serially diluted to give concentrations ranging between 1600-12.5 pg/10 µl and stored at -20 ^{o}C. 10 µl was used in each duplicated assay tube.

d) Labelled cortisol and antibody.

^{3}H-(1,2,6,7,) -Cortisol (Amersham, 250 µCi in 25 ml toulene/ethanol (9:1) solution) was diluted in 25 ml toluene/ethanol and stored at -20 ^{o}C. 100 µl of this stock was evaporated to dryness and resuspended in 20 ml PBSG. 100 µl stock antibody was added to 20 ml PBSG solution. The antibody (Cortisol R5) was raised by Dr. Gilham

and stored at -40 ^{o}C at 10 fold dilution in PBSG. Thus, a mixture of cortisol and antibody in 200 µl volume was added to each assay tubes.

e) Separation of bound and unbound Fractions.

After overnight incubation of tubes to ensure the binding of the ^{3}H-cortisol sample and sample cortisol to antibody, it was necessary to separate the bound fraction from unbound. This was carried out with dextran-charcoal solution, prepared by combining the following:

PBSG	100 ml.
Activated Charcoal	0.5 g.
Dextran	0.125g.

The solution was stirred on ice, for 0.5 hr before use.

f) Assay range.

The curve was useable between 25 pg and 1600 pg (Fig. 2.1).

α-Melanocyte-Stimulating-Hormone (α-MSH)

The plasma concentration of MSH was measured by a specific radioimmunoassay. Extraction of the hormone from plasma was described by Wilson and Morgan (1979). The assay technique was that described by Bowley et al., (1983) using the antibody R6FB at a final dilution of 60,000 (Gilham and Baker 1984). Synthetic α-MSH

Figure 2.1

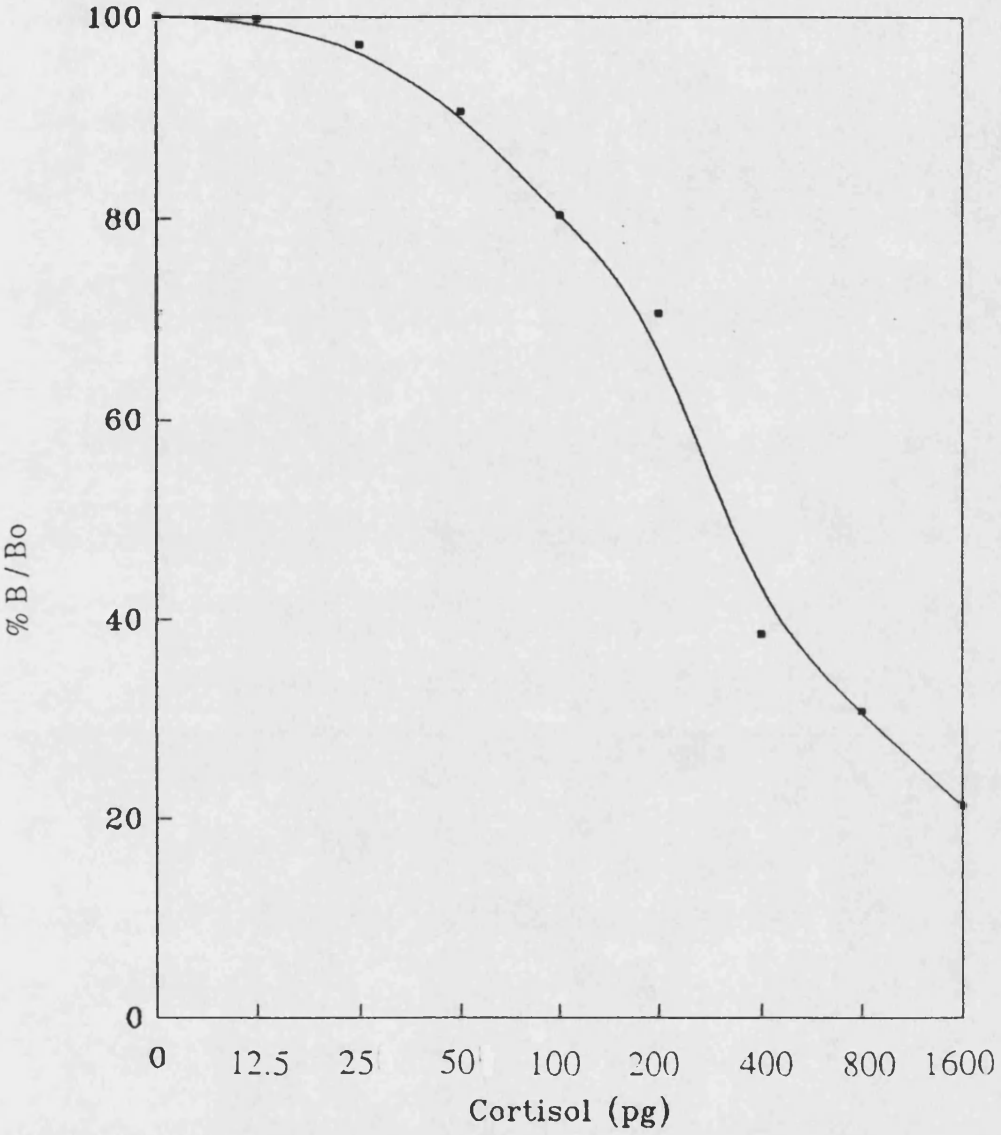


Figure 2.1. A typical standard curve for the measurement of cortisol in the charcoal-separated RIA.

(Sigma) was used as standard and ^{125}-I-labelled α-MSH was prepared as described by Wilson and Morgan (1979).

2.8 Statistics.

Results were expressed as means ± standard errors. The data were compared using one way analysis of variance (ANOVA) after checking for normal distribution. Logarithmic conversion was used when distribution was not normal.

CHAPTER 3

THE EFFECT OF DIFFERENT ACUTE AND CHRONIC STRESSES ON INNATE IMMUNE RESPONSE IN FISH.

3.1. INTRODUCTION

It is well established that environmental stress can trigger the outbreak of infectious diseases in fish (Fries, 1986; Angelidis et al., 1987; Maule et al., 1989). Fish grown in intensive culture system are frequently subjected to stressors such as handling, sorting, grading, transport, and poor water quality including low oxygen or high ammonia concentration. All these treatments represent stressful conditions to the fish, with subsequent physiological changes that often include immunosuppression and an increased susceptibility to infection (Wedemeyer, 1976; Barton et al., 1980; Maule et al., 1987; 1989).

In fish, stress related changes have been shown to affect non-specific immune responses such as phagocytosis (Weeks and Warinner 1984; 1986), or the production of hydrogen peroxide (Angelidis et al., 1987) and superoxides which constitute the respiratory burst (Bayne and Levy 1991a, b), or the production of lysozomal enzymes such as hydrolyses (Mock and Peters 1987) and also the production of various complement factors (Kaige et al., 1990). Also affected are inflammatory responses (MacArthur et al., 1987), and humoral immune responses including changes in lymphocytes in the circulation (Ellsaesser and Clem 1986; Barton et al., 1987; Pottinger and Pickering 1992). Apart

from the effect of stress, the effect of heavy metals such as cadmium, copper (Mushike et al. 1985; Thuvander et al., 1989) and drugs such as tetracycline, oxytetracycline, normally used in aquacultural practices to control bacterial and fungal infection also depress non-specific immunity.

Many of the techniques used to monitor these multiple responses listed above may demand expensive equipments. For instance, measurements of chemiluminiscence which reflects the respiratory burst. There is clearly need for a sensitive but easy method to monitor innate immune parameters. Moreover, most of the work on the innate immune system in fish and its response to stress has concentrated on long-term stresses. For example, Weeks and Warinner (1984; 1986) have demonstrated a decrease in macrophage phagocytosis in fish collected from highly polluted rivers which they had inhabited, one presumes, for a long period. However, there are no comparable studies on the effects of brief stresses on innate immune response. The only immediate response of macrophages to a stress-mediators is the work of Bayne and Levy (1991a) who monitored *in vitro* changes in the respiratory burst in response to catecholamines.

Therefore, an attempt have been taken to establish a cheap and easy method of monitoring phagocytic activity of macrophages, and to determine how this is influenced by stress, administered for periods ranging from a few minutes to 3 hours and whether the mild stresses are able to influence the innate immune responses or whether they need to be applied for a prolonged period to have any effect.

3.2. MATERIALS AND METHODS

3.2.1. Fish:

Rainbow trout (*Oncorhynchus mykiss*) weighing 220-325 g were obtained from commercial fish farm. They were kept in white 250 l capacity tanks with running water at 11 °C, and photoperiod of 18h light:6h dark. The fish were fed on commercial pellets and left to acclimate to aquarium conditions for at least two weeks before the experiments, unless otherwise stated.

3.2.2. Administration of chronic stresses.

A sample of 20 fish were collected from the trout farm, of which 5 were killed by a blow to the head 3 hours after collection (Acute stress). The remaining were maintained under aquarium conditions in white tanks for 6 days. Of these, 5 were left undisturbed (Control), 5 were injected daily with 1 ml saline (0.8% NaCl) into a peritoneal cavity (Chronic stress), 5 were injected daily with 1 ml saline containing 0.2 mg/ml dexamethasone (Chronic plus dexamethasone treatement). These fish were killed, bled and assayed for macrophage phagocytosis 2 h after the final injection.

Another group of fish were collected from farm and acclimated to aquarium conditions for >2 weeks. Of these, some were chronically stressed by noise plus confinement or exposing them to twice daily to one and half inch low water, over 6 days. Control fish were left undisturbed. Fish were killed 3h after the last stress.

3.2.3. Administration of mild acute stress.

To deliver a mild stress, fish were anaesthetised with or without phenoxyethanol (6:10000 v/v) and injected intraperitoneally (ip) with 0.5 ml of 0.8% NaCl solution. They were returned to their home tank and killed 3h later.

3.2.4. Administration of intense acute stress.

A more intense stress was delivered by placing the fish in a black plastic dustbin, half filled with 80 l of water, and switching on a small electric aerator motor which vibrated against the side wall of the tank. This noise vibration stress was given for 1h and the fish were left for another 2h in the same dustbin as a confinement stress and killed after 3h of stress.

3.2.5. Macrophage Phagocytosis Assay.

Briefly, at the time of autopsy, fish were caught with one sweep of the net and anaesthetised deeply. The spleen and pronephros tissues were removed and forced individually through a nylon tea strainer into 4 ml of Leibovitz medium (L-15, Sigma chemical Co., Poole, Dorset) containing 0.1 % fetal bovine serum and 4U/ml of sodium heaparin. (Grade II, Sigma). The pronephric cell suspension was diluted a further 4-folds as pronephros contain more cells than the spleen. 2 ml of this pronephric or spleen suspension were flooded onto individual, pre-washed microscope slides and the phagocytic cells were allowed to adhere for 90 min. at room temp. (approx. 22-25 °C).

Non-adherent cells were washed off in 0.15 M Phosphate Buffered Saline (PBS, pH 7.8) and the attached phagocytes were flooded with yeast cells in L-15 medium containing 4% bovine serum.

Phagocytosis was allowed to proceed for 90 min., after which the slides were rinsed in 0.02 M PBS, pH 7.8, fixed in methanol for 10 min stained with May/Grunwald stain. The cells were examined under oil immersion.

The phagocytic index (P.I.) of macrophages were determined by counting the average number of yeast cells phagocytosed by macrophage from each fish. From these counts was also determined the percentage of macrophages which engulfged one or more yeast cells (% phagocytosis).

3.2.6. Determination of plasma cortisol.

About 2-3 ml blood was collected from fish containing 50 µl 6% EDTA disodium salt as a anticoagulant. After centrifugation at 3000 x g for 15 min., aliquots of plasma were stored at -20 °C untill assay. Cortisol concentration was determined by radioimmunoassay (Rance and Baker, 1981); dexamethasone does not interfere with the recognition of cortisol showed 1% cross-reactivity in the assay. Tritiated cortisol was used as standard.

3.3. RESULTS

3.3.1. The effect of different chronic stresses on macrophage phagocytosis.

After staining with May-Grunwald/Giemsa, two types of phagocytic cells were observed. One cell type, believed to be a macrophage, had darkly staining cytoplasm and a rounded nucleus (Mac Arthur et al., 1984) (Fig. 3.0). A second type, with chromophobic cytoplasm and a more irregularly-shaped nucleus, may have been a neutrophil. In several experiments described below, the phagocytic index (P.I.) of both cell types was determined. The paler, putative neutrophils engulfed comparatively less yeast cells and did not respond to stress (Table 3.1) although macrophages showed a strong response. Only macrophages were examined in later experiments.

The preliminary experiment was designed to investigate the effect of transporting fish from the farm to the aquarium (acute stress) and the effect of subsequent chronic stress (Table 3.2A). The average number of yeast cells engulfed per macrophage from a total sample of 200 macrophages from each fish, which is called phagocytic index (P.I.), was very low after 3h of transport (Table 3.2A) ($P<0.001$). The percentage of macrophages which phagocytosed one or more yeast cells (% phago.), was similarly depressed within 3h.

To test the effect of repeated stress on the ability to recover from transport stresses, fish were given a daily injection of saline or of dexamethasone for 6 days, or left undisturbed. Although this experiments was done by Mike Tomlinson, the results are described as

Figure 3.0

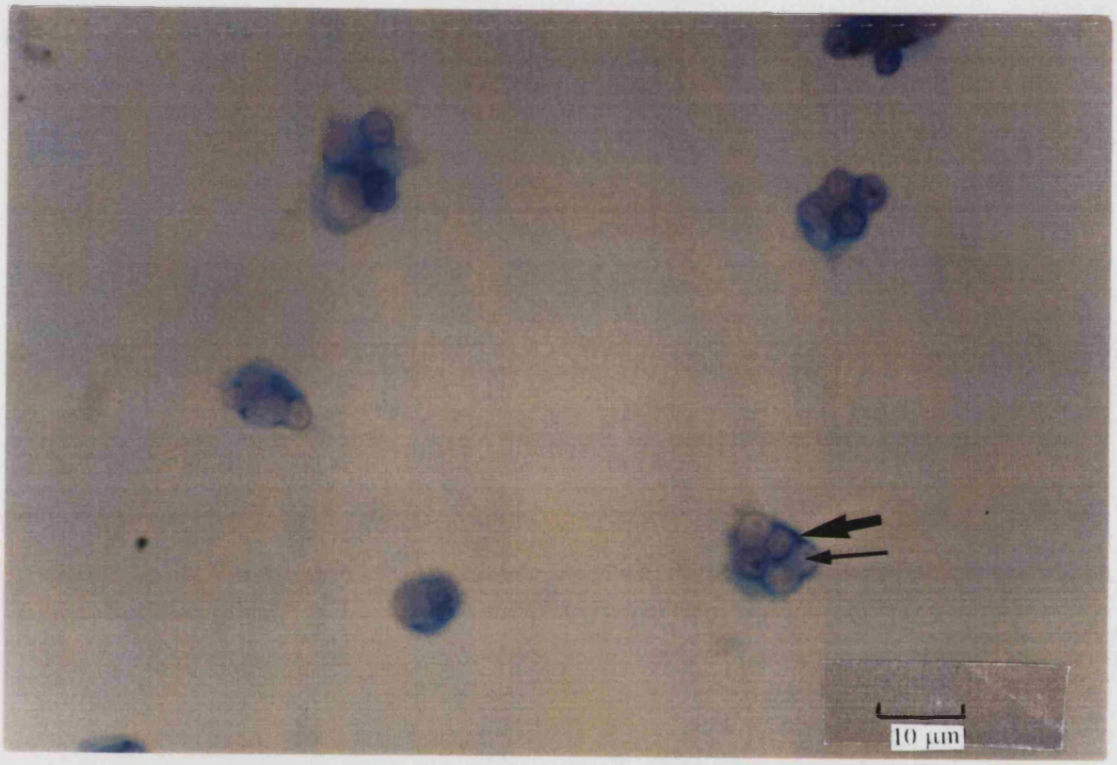


Figure 3.0 Phagocytosis of yeast cells (small arrow) by trout pronephric macrophages (big arrow) (stained with May-Grunwald /Giemsa stain). (The scale bar is 10 µm)

Table 3.1

The effect of intense acute stress on neutrophil phagocytosis

TREATEMENT	PRONEPHROS		SPLEEN		CORTISOL (ng/ml)
	% PHAGO.	P.I.	% PHAGO.	P.I.	
NEUTROPHILS					
CONTROL	75±4.5	1.7±0.09	63±8.6	1.7±0.11	3±1.3
NOISE STRESS	76±5.8	1.7±0.23	69±8.4	1.9±0.32	34±13.0*
MACROPHAGES					
CONTROL	97±1.3	3.3±0.13	95±3.4	3.3±0.20	3±1.3
NOISE STRESS	72±3.9**	1.8±0.13**	82±4.4*	2.0±0.19**	34±13.0*

Table 3.1. Comparison of neutrophil and macrophage phagocytic activity in response to intense acute stress. 200 cells from individual fish were assessed.

(n=5). * $P<0.05$ ** $P<0.01$ compared to control.

they are directly pertinent to these studies. Macrophages from the control fish showed a significant recovery of the P.I. from the stress of transport, which was partially prevented by stress of a daily saline injection (chronic) (Table 3.2A). The corticosteroid analogue, dexamethasone, further suppressed recovery of phagocytic activity, such that macrophages from these fish had a phagocytic index lower than chronically stressed fish. The ranking order of phagocytic index for kidney macrophages was: control > saline > dexamethasone injection > acute stress. This ranking order was the same for splenic macrophages. Acute and chronic stress resulted in similar plasma cortisol values at the time of autopsy (Table 3.2A).

Daily injection for 6 days in another experiment depressed the splenic but not pronephric phagocytic index (Table 3.2B) ($P<0.02$) as compared to acute stress. However, in another experiment, daily noise plus confinement stress was equally suppressive to pronephric and splenic macrophages (Table 3.2C).

The effect of twice daily low water stress over 6 days was investigated as a third type of chronic stress, which was easily administered (Table 3.2D). This type of stress neither altered the phagocytic index nor the percentage phagocytosis. The plasma cortisol levels were significantly raised in response to acute, transport plus injection or only injection and failed to raised in response to low water stress, which was also presumably inadequately stressful to cause any physiological response.

3.3.2. The effect of a mild acute stress on macrophage phagocytosis.

It was observed that the chronic stresses administered above seemed to be less detrimental over a 6 day period, than the 3h transport stress. Therefore, how acute

Table 3.2.

The effect of different stresses on macrophage phagoytosis.

TREATMENT	PHAGOCYTIC INDICES				CORTISOL
	PRONEPHROS		SPLEEN		(ng/ml)
	% PHAGO.	P.I.	% PHAGO.	P.I.	
A. CONTROL	96±0.4	5.9±0.26b	78±4.1	4.9±0.29b	1.6±0.4
ACUTE (Transport)	75±4.4*	2.6±0.16**a	85±2.0	3.0±0.18**a	44±3.0*
CHRONIC (Daily inject.)	94±2.8	4.8±0.22*c	84±7.0	4.2±0.20b	51±6.0*
DEXA. (Daily inject.)	91±1.8*	3.8±0.25**d	79±3.2	3.0±0.32**	1±0.1
B. CONTROL	94±1.2	3.3±0.14	76±6.9	3.7±0.21	1.2±0.1
CHRONIC (Daily inject.)	88±2.2	3.0±0.16	64±11.0	2.4±0.19**	54±21*
C. CONTROL	96±0.68	3.8±0.03	91±2.5	3.8±0.18	8±3.3
CHRONIC (Daily noise)	88±3.7*	3.2±0.19*	85±6.1	2.9±0.31**	3±0.78
D. CONTROL	94±2.5	2.9±0.18	90±3.5	3.0±0.18	3.0±0.94
CHRONIC (Twice daily low water)	94±2.2	3.3±0.05	87±1.98	2.8±0.07	7.5±2.0

Expt. 2A. Fish were collected from a fish-farm and some were killed on arrival at the aquarium, (Acute stress); the remainder were kept in the aquarium for a further 6 days. Of these, five were chronically stressed by a daily injection of 1 ml saline (Chronic stress); steroid-treated fish were injected daily for 6 days with 0.2 mg/ml dexamethasone (Dexa.). Fish were killed 2h after the last injection.

In another expts. **2B, C & D.** Fish were either reared in the aquarium (Expt.C) or collected from a farm fish and acclimated to aquarium conditions for > 2 weeks (Expt. B & D) before being stressed daily, as indicated. Stress was applied over 6 days and fish were killed 3h after the initiation of stress.
* $P<0.02$ ** $P<0.001$ compared to controls (n=5).

Values in the same column with different superscript letters are significantly different from each other.

stress affects the phagocytic activity of macrophages was assessed in subsequent experiments. Fish were exposed to a mild, brief stress consisting of a saline injection with or without anaesthesia. The mean number of yeast cells macrophage phagocytosed in a total population of 200 macrophage (P.I.) was significantly reduced by simply catching the fish from their home tank and injecting them with saline either under mild anaesthesia ($P<0.001$; Table 3.3A & B) or without anaesthesia ($P<0.001$, Table 3.3C). This treatment also depressed the % phagocytosis ($P<0.02$, Table 3.3A-C). This reduction was seen within 3h of the stress and both pronephros and spleen phagocytes responded similarly. The plasma cortisol was significantly raised in response to this stress. In other words, a brief stress rapidly increased the number of inert macrophages which engulfed no yeast cells (Fig. 3.1). Ignoring the inert macrophages which phagocytosed nothing, the average number of yeast cells taken up by active macrophages were similarly depressed within 3h of stress (Fig. 3.1).

3.3.3. The effect of intense acute stress on macrophage phagocytosis.

A mild acute stress was equally depressive to pronephros and spleen macrophages. Whether more prolonged stress affects the phagocytic activity of macrophages more strongly was investigated next. A more prolonged stress was designed in which fish were stressed by placing them in a black dustbin and exposing them to 1h noise vibration and further 2h of confinement in the test tank to mimic transport conditions. This stress, which was repeated in several experiments, also significantly reduced both the phagocytic index and % phagocytosis in the pronephros and spleen (Table 3.4 A-D) (Fig. 3.2) respectively.

Table 3.3

The effect of a mild acute stress on macrophage phagocytosis.

TREATMENT	PHAGOCYTIC INDICES				CORTISOL
	PRONEPHROS		SPLEEN		(ng/ml)
	% PHAGO.	P.I.	% PHAGO.	P.I	
A.CONTROL	93±0.9	3.6±0.08	97±1.0	4.1±0.22	6.3±0.9
INJECT.	78±3.2**	2.3±0.11**	82±2.7**	2.6±0.09**	20±2.8**
B.CONTROL	96±1.72	3.4±0.09	———	———	1±0.05
INJECT.	81±3.5*	2.3±0.17**	———	———	17±11.9**
C.CONTROL	97±0.7	4.1±0.06	99±0.6	4.4±0.20	3±3.3
INJECT.	81±5.0**	2.5±0.25**	74±3.3**	2.8±0.21**	28±6.7*

Table 3.3 Experiments A-C are replicate in which fish were stressed by a saline injection given mild anaesthesia (Expt. A & B) or without anaesthesia (Expt. C). They were returned to their home tanks and killed 3h later. Phagocytic index was determined from two hundred macrophages per fish.

(n=5). * $P<0.01$ ** $P<0.000$ compared to controls.

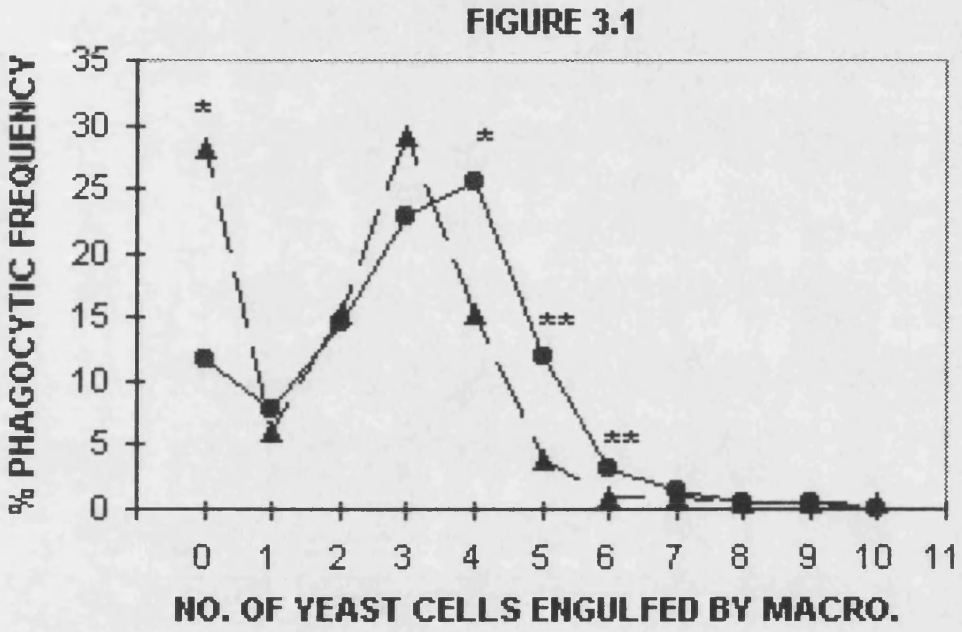


Figure 3.1. The percentage frequency distribution of yeast cells phagocytosed by pronephric macrophages from control fish (circle and solid line) or acutely stressed fish (triangle and dotted line). Two hundred macrophages were counted from each fish. One-way ANOVA.

n=5; * $P<0.05$ ** $P<0.01$ compared to stressed fish.

Table 3.4.

The effect of intense acute stress on macrophage phagocytosis.

TREATMENT	PHAGOCYTIC INDICES				CORTISOL
	PRONEPHROS		SPLEEN		(ng/ml)
	% PHAGO.	P.I.	% PHAGO.	P. I.	
A. CONTROL	88±2.7	3.1±0.11	92±3.5	3.2±0.26	<1.0
NOISE STRESS	72+5.4*	2.2+0.17**	78+3.3*	2.3+0.18*	34±13**
B. CONTROL	97±1.3	3.3±0.13	95±3.4	3.3±0.20	3±3.3
NOISE STRESS	72±3.9**	1.8±0.13**	82±4.4*	2.0±0.19**	35±13**
C. CONTROL	96±0.68	3.8±0.03	91±2.4	3.8±0.21	8.2±3.3
NOISE STRESS	81±3.7*	2.4±0.10**	69±5.9**	2.4±0.24*	27±6.1*
D. CONTROL	70±5.7	1.9±0.28	----	----	<1.0
NOISE STRESS	56±1.9*	1.3±0.09*	----	----	15±3.**

Table 3.4. Experiments A-D are replicate in which fish were stressed for 1h noise plus 2h confinement (intenese acute stress). They were returned to home tank and killed 3h later. Two hundred macrophages were observed under microscope to determine phagocytic index per fish.

* P<0.05 ** P<0.01 compared to controls.

FIGURE 3.2

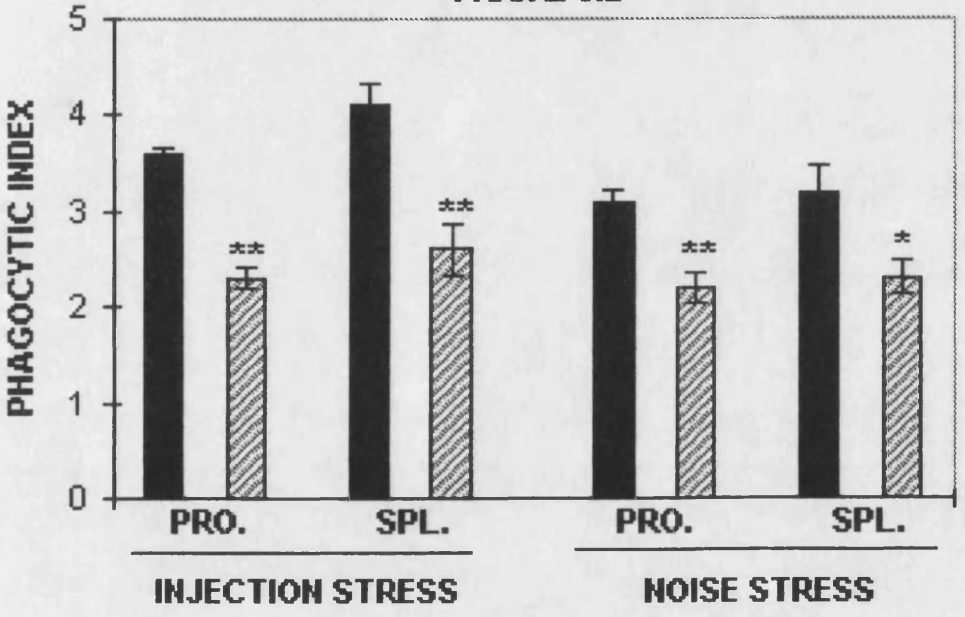


Figure 3.2. The influence of mild and intense acute stress on pronephric and splenic macrophage phagocytic index determined 3h after the initiation of stress. Control values are shown by solid bars; stress values are shown by hatched bars. Mean values ± sem.

n=5; * $P<0.05$ ** $P<0.01$ compared to control values.

To compare the responses to mild, 3h and one week of stress, the P.I. of experimental fish was expressed as a percentage of the P.I. in control fish. mild and intense acute (3h) stress were equally suppressive to phagocytic activity (Table 3.5B & C) whereas this activity tended to recover during 6 days of repeated stress (Table 3.5A). In every case of acute stress (mild or intense), a significant depression in % of phagocytosis was seen, but this was not always noticeable in some experiments when stress was repeated for 6 days.

3.3.4. Phagocytes adherence.

Because of the P.I. would appear depressed if active macrophages in the pronephros and the spleen were to lose their adherent capacity and moved out of these tissues, an attempt was made to determine the adherence of the macrophages from 3 experimental conditions. The total number of macrophages adhering to the slide was counted from five different areas of slides from two experiments in which fish had been injected with saline, or with cortisol or subjected to low water stress (control experiments which did not influence P.I.) both of which suppressed the P.I. The results, presented in table 3.6, showed that the number of macrophages adhered to glass slides was not apparently affected by stress or by raised plasma cortisol concentration.

Table 3.5.

Comparison of phagocytic suppression by acute and chronic stresses, expressed as percentage of control values.

TREATMENT	PRONEPHROS		SPLEEN	
	% PHAGO.	P.I.	% PHAGO.	P.I.
CHRONIC STRESSES				
A. 6d Injection (Table 3.2A)	98	81.3*	107	98
6d Injection (Table 3.2B)	93.6	90.9	84.2	64.9**
6d Noise (Table 3.2C)	91.7*	84.2*	93.4	76.3**
6d Lower water (Table 3.2D)	100	121	97	93
		94.35+9.1		83.1+7.6
B. MILD ACUTE STRESS				
Injection (Table 3.3A+Ana)	84**	63.9**	84.5	63.4**
Injection (Table 3.3B+Ana)	84**	67.6**	—	—
Injection (Table 3.3C)	84**	61.0**	75**	63.6**
		64.2+1.9		63.5+0
C. INTENSE ACUTE STRESS				
Noise 3hr. (Table 3.4A)	82*	71**	85*	71.9*
Noise 3hr (Table 3.4B)	74**	54.5**	86*	60.6**
Noise 3hr. (Table 3.4C)	84	63.2**	75.8**	63.2*
Noise 3hr. (Table 3.4D)	80*	67.0*	—	—
Transport (Table 3.1A)	78*	44.1**	109	61.2**
		60±4.8		64.2±2.62

Table 3.5. Values show the activity if macrophages from stressed fish, calculated as a % of their activity in control fish from the same experiment. n= 4-5 for each experiments.
* Values significantly differrent from control. Ana= anaesthesia.

Table 3.6.

The effect of different experimental conditions on macrophage adherent capacity.

	Number of adherent macrophages		
	PRONEPHROS	**SPLEEN**	**CORTISOL (ng/ml)**
CONTROL	41.6 ± 3.6	9.9 ± 1.0	6.3 ± 0.86
INJECTION STRESS	66.3 ±12.3	9.0 ± 1.3	20.1 ± 2.84
CORTISOL INJECTION	34.7 ±6.3	7.9 ± 1.3	59.8 ±7.24
CONTROL	16.0 ± 2.7	3.4 ± 0.4	3.0 ± 0.94
CHRONIC STRESS	18.6 ± 2.2	4.9 ± 1.0	7.5 ± 2.0

Table 3.6 As an index of macrophage adherence, the number of macrophages within a high-power field of view was counted from 5 regions on a slide, to obtain an average values for each fish.

Five fish were investigated for each experimental condition.

Values are means ± sem (n = 5 fish).

3.4. DISCUSSION

The activity of macrophages can be assessed by a number of methods, based on the different facets of their physiological repertoire. Many methods that have been applied to fish studies have monitored some aspects of the microbicidal capacity of the cells, for instance, the production of reactive oxygen species such as O_2^- and H_2O_2 measured by chemiluminiscence (Ellsaesser and Clem 1986; Chung and Secombes, 1987; Angelidis et al., 1987; Whiskovsky et al., 1987; Flory and Levy 1991a, Bayne and Levy 1991b) or the level of the lysozomal enzymes, acid phosphatase (Secombes, 1986; Chung and Secombes, 1987). All these methods have been used for various purposes. Ellsaesser and Clem (1986) and Angelidis et al., (1987) have used for haematological and immunological changes in response to stress. While Bayne and Levy (1991a,b) and Whiskovsky et al., (1987), monitored the *in vitro* effects of catecholamines and also drugs such as tetracycline, oxytetracycline used in aquacultural practices on macrophage activity.

The present study demonstrates one of the most easily observed events- phagocytosis- shown by macrophages. Other studies using this criterion have expressed phagocytosis simply as the percentage of cells which show any phagocytic activity. This is satisfactory in cases involving a very marked depression of macrophage activity. For example, Weeks and Warinner (1984; 1986) have reported a 3-4 fold decrease in percentage of cell showing phagocytosis in the spot, *Leisostomus xantharus* and the hogchoker, *Trinectes maculatus*, from polluted rivers; but this method of assessment may be rather insensitive in less stressful situations.

This chapter describes a novel method of expressing macrophage phagocytic activity in terms of the phagocytic index (P.I.), as well as the percentage of phagocytosis from the total population of macrophages. This method of assessment was easy and more sensitive, sometimes revealing a significant change in phagocytic activity even when the percentage of phagocytosis was not significantly altered (Table 3.2). Macrophages respond to stress in two ways: in many cases, there is an increase in the percentage of cells which fail to engulf any yeast cell during the experiment (Fig. 3.2). However, the percentage of phagocytosis is not always significantly depressed, but there is a decline in the mean number of yeast cells which are phagocytosed i.e. the P.I. It is for this reason that the P.I. is a more sensitive measure of macrophage activity than is the percentage phagocytosis. This method can also be use easily in field studies, requiring little equipment other than nylon tea strainers, glass slides and stain.

Both macrophages and neutrophils have been shown to attach to glass or plastic surfaces (Cohn, 1968). Using this property to isolate these cells, two types of phagocytic cells were observed. One cell type, believed to be a macrophage, had darkly staining cytoplasm and a rounded nucleus (MacArthur et al., 1984). A second type, with chromophobic cytoplasm and an irregularly-shaped nucleus, may have been neutrophils.

Using this method of assessment, the phagocytic activity of these two cell types were investigated in response to different stresses.

The phagocytic activity of neutrophils have been studied by many authors, using cells from different sources such as peritoneal cavity in plaice (Ellis et al., 1976; MacArthur et

al., 1984) and blood in catfish (Ellsaesser et al., 1985; Finco-Kent and Thune 1987). Phagocytic activity of neutrophils studied in this chapter was comparatively less intense than for macrophages and was not influenced by stress (Table 3.1). No comparable data in the literature are available on stress-induced changes on neutrophil phagocytosis in fish. There are relatively few neutrophils in the pronephros and spleen (Congleton et al., 1990) and it may be that those found in these tissues are immature, and not yet fully phagocytic, possibly being released into the blood at their maturation. In mammals, neutrophils only reach full phagocytic activity after stimulation by inflammatory cytokines, when they also display increased adhesiveness and migrate to sites of inflammation. Anderson et al., (1992) working on rainbow trout, also found that neutrophil adhesiveness and phagocytic activation was enhanced by infection. Because of their lesser abundance and activity in pronephric and splenic extracts, and their apparent unresponsiveness to stress, neutrophils were not investigated further in these studies.

Using the phagocytic index method, the effect of very brief stress was investigated. Fish exposed to a very brief injection stress given with or without anaesthesia, showed a suppressed P.I. within 3h (Table 3.3). The depression seen after this stress was apparently not much affected by anaesthesia, but may be due to the combination of catching and injecting the fish. Pollock et al., (1987) also have found no change in NK cell activity in response to anaesthetic treatment.

Thus it was observed that simply catching the fish and injecting them was a strong enough stress to depress P.I. Therefore, to see if greater stress can cause a greater decline in P.I., a potentially stronger stress was applied: Fish were exposed to 3h

confinement in 80 L of water (16L/fish) including 1h of noise. The P.I. of the macrophages was significantly depressed in response to this stress but the depression was of a similar magnitude to that caused by injection stress (Table 3.5). Although more prolonged, confinement and noise did not cause a greater rise in plasma cortisol than a brief single stress (Table 3.3). It may not have been more stressful. The variation in responsiveness of different batches of fish makes exact comparison between experiments difficult.

The influence of either more prolonged or a brief acute stress on decreased phagocytic activity of macrophages, seen within 3h of these stresses, was to reduce the number of phagocytic events. The time course of macrophage responsiveness has not been investigated previously. The similar rapid response to stress has been observed on other aspects of fish immune system. For instance, antibody production by lymphocytes (Maule et al., 1989) was significantly depressed within 4 hr of acute stress, and the chemiluminiscence response of head kidney phagocytes was significantly depressed within 30-60 seconds of applying catecholamines (Bayne and Levy 1991b). Similarly, in mammals, a rapid response to stress has been observed on other immune cells, such as natural killer cell activity which was significantly reduced 2h after footshock (Jain et al., 1991), surgical stress in rats (Pollock et al., 1987) or 3h of restraint in monkeys (MorrowTesch et al., 1993) and blastogenesis was reduced within 15 min. of catecholamine administration (Crary et al., 1983). All these are examples of decrease in activation of cells which would have been more active before stress. It implies an immediate effect of neurotransmitters or hormones acting on the immune cells to depress their former activity.

In considering why a reduction in P.I. might occur, it is necessary to discuss first how one should interpret the change in P.I. A decline in P.I. may be attributable to a real decrease in the activity of macrophages, or to a removal of active macrophages to some other site of the body such as blood, intestine, thymus or general body stroma, leaving only phagocytically less active ones in the pronephros and spleen. This depletion does not necessarily involve an actual migration of cells out of the pronephros and the spleen into these sites, but could also arise from a loss of adhesiveness of the macrophages so that they no longer attach to the glass slide. Inspection, the number of macrophages adhering to the glass slide showed no change in response to stress or increased plasma cortisol (Table 3.6). However, macrophage counting on glass slide may not be a satisfactory measure, as macrophages were not uniformaly attached to slides. It is possible that increased plasma cortisol may have an effect on macrophage adhesiveness when applied for prolonged period. Thus prolong dexamethasone treatment reduced the number of macrophages in the spleen (Tomlinson, report).

However, although a certain change of macrophage distribution within the body could partially contributed to a change in the percentage of phagocytosis and P.I. after stress, this could not completely account for the level of change recorded here. Thus inspection of tables 3.3 & 3.4 shows that the percentage of inert macrophages, which engulf no yeast cells at all, increases after stress up to 9 fold, with an average increase of 4 fold over separate experiments (from 5.5 ± 1.4 to 22.5 ± 1.8 (n=6) % inert macrophages) separate experiments. It would require a massive emigration of 80% of the initial active macrophage population to achieve this increased % in inert cells, were no reduction in the activity of individual macrophages to occur. Thus, while loss of phagocytically

macrophages from the pronephros and spleen or a decline in their adhesiveness to the glass slide may occur, it is unlikely to be the sole cause of the decline in P.I. A change in immune cell distribution is discussed further in chapter 6.

Decreased P.I. in response to stress applied in this study may also be caused by other stress agents such as, catecholamines or other hormones/neurotransmitters, eg. CRH, ACTH, NPY, VIP, neurotensin, substance P, vasopressin, β-endorphin etc. In mammals, many of these agents have been shown to be released from nerve terminals, peripheral tissues and blood cells and affect many immune functions. How these might directly influence phagocytosis is uncertain. One possibility is a decreased recognition of opsonizing factor on the yeast cell. The importance of such opsonizing factors is seen in table 3.4D. In this experiments, both controls and stressed fish showed very low phagocytic activity. The reason for this was that in this experiment, exceptionally, only 0.1% instead of the usual 4% fetal calf serum as an opsonizing factor. Interestingly, however, the % reduction of the P.I. is normal.

Brief or continuous stresses applied in this study over 3 hr found to be equally supressive to macrophage phagocytic activities. Therefore, it was of interest to investigate to what extent macrophage phagocytic activity would be affected if these stresses here applied repeatedly for period such as over a week. The preliminary investigation showed that macrophage phagocytic activity in the pronephros and spleen was less significantly depressed when fish were exposed to repeated stress for 6 days than when the same stress applied for the first time. Thus, assessment in table 5, an injection caused phagocytic activity to decline to 64% (pronephros) or 63% (spleen) of controls when

administered once only, but when administered on 6 successive days, the P.I. was reduced to only 86% and 81.5% of controls in the pronephros and spleen respectively. Similarly the stress of noise and confinement caused a decrease in P.I. to 64% and 65% of control values in to the tissues, the first time it was administered but this recovered to 84% and 76% of control values after 6 days of daily noise and confinement (Table 3.5). Thus, repeated stress is less depressive than an acute stress.

If phagocytic activity is directly impaired by stress "messengers" (either hormones or neurotransmitters) depressing the activity of cells, rather than causing their relocation, the continuous release of these messengers might cause receptor let-down on the leucocytes, so that the depressive effect to respond is decreased. Alternatively, work on mammals suggest that, rather than a loss of receptors, continued stress leads to a decline in the release of messengers such as ACTH, β-endorphin and corticosterone (Collector 1977; Irwin and Haugher 1988). Thus, Irwin and Haugher (1988) have shown that splenic NK activity was decreased in response to a period of daily immobilization stress for 4 days. but when stress was repeated for 10 days, the levels of hormones such as β-endorphin and ACTH were returned to basal level, while the NK cells also returned to normal. However, in the present study, there was no evidence from cortisol measurements that the neuroendocrine axis had been downregulated, although, individual components of the HPA axis or the CNS may have been reduced. Thus, a loss of responsiveness by immune cells seems a likely explanation.

If the decline in P.I. is attributable to a removal of active macrophages, it is equally possible that their response to stress-mediators, which caused a loss of adhesiveness or

resulted in their active migration out of the tissue, is now less marked and that the cells had returned to their former sites and adhesiveness. It is also possible that stress directly or indirectly might induce blastogenesis such that newly formed active macrophages are added to the pronephros and splenic population. This point will be discussed in a later chapter (chapter 6).

Fish were given dexamethasone to determine if this cortisol analogue would mimic the effect of endogenous release of cortisol by stress. Dexamethasone caused suppression of P.I. more than acute stress (Table 3.4A). The role of endogenous cortisol released during acute stress, is investigated in chapter 5.

In summary, this chapter describes:

(1) A simple method of assessment of phagocytic activity. This phagocytic index is sometimes more sensitive and descriptive than the previously applied measure of % phagocytosis.

(2) Using this P.I., the work show that a brief handling stress, causes a significant reduction in P.I. measured 3h later. A continuous 3h stress causes in P.I. of similar magnitude.

(3) When these stresses are repeated daily for 6 days, the decline in P.I. is less marked, indicating an adaptation to the stress.

(4) The interpretation of what causes a reduction in P.I.- a real reduction in phagocytic activity or a change in the population of macrophages being studied due to the relocation of active macrophages, is discussed.

CHAPTER 4.

RECOVERY OF MACROPHAGE PHAGOCYTIC ACTIVITY FROM ACUTE STRESS AND ITS ENHANCEMENT FOLLOWING THIS STRESS

4.1. INTRODUCTION

It has been long recognised that stress and resistance to disease are related. Stress causes many changes in the physiological systems of the body, including the defence system and modulates many different mechanisms, suppressing some and exaggerating others. This may be advantageous or injurious to the animals health concerned. The complex interaction between the stress factors and the animal's physiological states will eventually determine their ability to overcome infections.

If stress is for a short duration, all pathophysiological changes return relatively quickly to normal (Fehr et al., 1988; 1989; Aastrad et al., 1991) but if the challenge is continuous these changes last for a long time and may result in deterioration of the animal's health (Aastrad et al., 1991).

In mammals, stressful events suppress a broad spectrum of both humoral and cellular immunological responses. However, stress is not always immunosupprssive. The influences of stressors on immune responses are complex and depend not only the characteristics of the stressor but also its nature, magnitude, duration and the initial condition of the animals (Mojan and Collector 1977; Blecha et al., 1982; Lysle et al.,

1987). Some stressors such as exercise (Blecha et al., 1982; Fehr et al., 1988; 1989; Ortega et al., 1993), hot or cold shock (Aastrad et al., 1991), electrical footshock (Jain and Stevenson 1991), immobilization (Blecha et al., 1982), sound (Mojan and Collector 1977) and osmotic and physical stimulation (Fujiwara and Orita 1987) have been shown to increase cellular (Blecha et al., 1982; Jessop et al., 1987; Jain and Stevenson 1991; Wood et al., 1993) and humoral (Fujiwara and Orita 1987; Fehr et al., 1989; Aastrad et al., 1991; Wood et al., 1993) immune responses. However, only few authors have shown the enhancement in macrophage phagocytic activity and their enzymes in response to stress (Fehr et al., 1988; 1989; Sharp and Koutedakis 1992).

As in mammals, investigations in fish have demonstrated the stress-induced changes in humoral immune responses and their recovery from these stresses (Maule et al., 1987; 1989) But, there are no comparable data available on immuno-enhancement following challenging stress. Similarly, stress-related changes in the innate immune response have been shown by a few authors (Peters and Schwarzer 1985; Angelidis et al, 1987) but again their subsequent recovery and enhancement has not yet been investigated.

Macrophages play an important role in the first line of defence in lower vertebrates including fish. In the previous chapter it was shown that even a brief handling stress influences the phagocytic function of the pronephros and spleen within 3h stress period. Therefore, it was of interest to investigate the time period required for macrophage phagocytic activity to recover from acute stress and whether immunoenhancement occured after stress

4.2. MATERIALS AND METHODS

4.2.1 Experimental design:

Fish were transferred from their home tank into a black dustbin and were stressed by 1h noise plus 2h further confinement stress (intense acute stress) as mentioned previously (chapter 3). Immediately after this, 5 fish were anaesthetised and killed, together with an undisturbed control group. Another 5 stressed fish were transferred to their home tank and left to recover for six days.

In other experiments, immediately after noise plus confinement stress, one group of fish was killed immediately and other groups of 5 fish were transferred to their home tank for 24 hr, 1 week and 2 weeks recovery periods. After each time period, they were sacrificed with their unstressed controls and macrophage phagocytic activity from the pronephros and the spleen was determined as described chapter 2.

4.3. RESULTS

The results presented in table 4.1 show that macrophage phagocytosis was significantly depressed in response to an intense acute stress within 3h ($P<0.01$) and remained depressed for 24 hr after this stress period ($P<0.01$; Table 4.1). The plasma cortisol level in control fish was 8.2 ± 3.3 ng/ml, and further raised in response to acute stressed to 20 ± 2.3 ng/ml. However, this depression in phagocytic activity of pronephric and splenic macrophages, seen in response to acute stress, was completely recovered after 6 days and remained the same for another week with the same plasma cortisol level as that seen in control (Control 2.7 ± 1.23 ng/ml and one week recovery 7.0 ± 0.41 ng/ml).

Surprisingly, six days after stress, the population of macrophages in the pronephros displayed a higher phagocytic activity in 3 out of 5 experiments, than in undisturbed fish ($P<0.01$; Fig. 4.1). This increase was significant in three experiments, and raised but not significantly so in another experiment (Table 4.1) due to a bigger standard error (Expt E). Responses by splenic macrophages were more variable and did not show significant enhancement in any experiment (Fig. 4.2).

Table 4.1

Effect of different post-stress periods on macrophage phagocytic activity.

TREATMENTS	PRONEPHROS		SPLEEN	
	% PHAGO.	P.I.	% PHAGO	P.I.
A. 3 HR	84.4*	63±2.4**	75.8**	85±2.8**
B. 24 HR.	85.4*	63±4.5**	——	——
C. 1 WEEK#	100	129±8.4**	98.9	113±11.0
D 1 WEEK	121	152±9.5**	——	——
E. 1 WEEK	100	116±11.1	83.5*	73±7.0*
F. 1 WEEK	109	90±6.7	——	——
G. 1 WEEK	99	121±6.2*	99.7	124±10.0
H. 2 WEEK	100	100±4.0	——	——

Table 4.1. Fish were stressed by 1h noise plus 2h confinement stress and their phagocytic activity was assessed at 3h, 24h, 1 and 2 weeks with their respective controls and was expressed as percentage of control values.

* $P<0.05$ ** $P<0.01$ values significantly different from control
#Controls were killed at the start of the experiment.

FIGURE 4.1

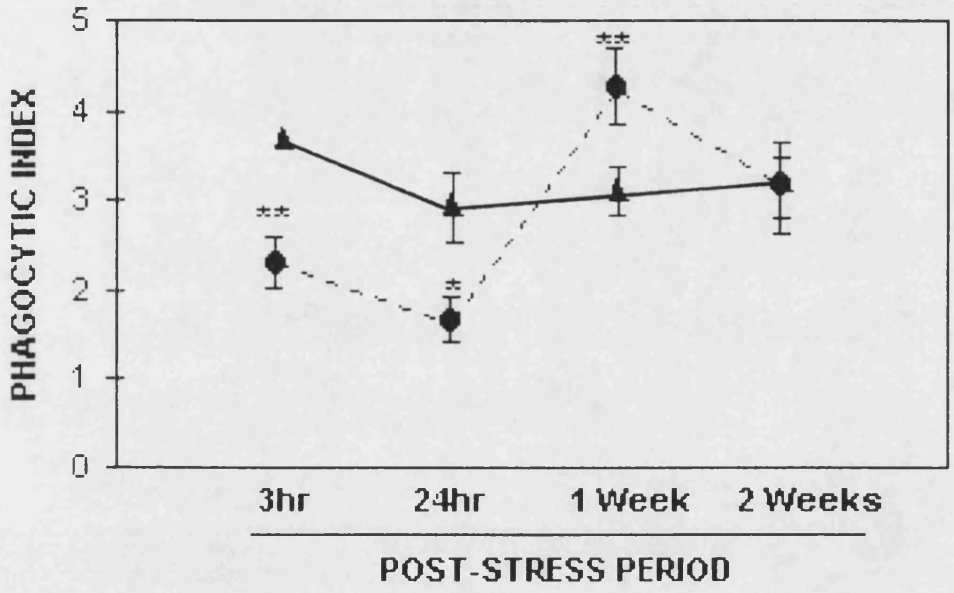


Figure 4.1. The effect of different post-stress periods on pronephric macrophage phagocytic index. Control values shown by triangle and solid line and post-stress values by circle and dotted line. The data pooled from expts. A, B, C, D and E.

n=5; * $P<0.05$ $P<0.01$ compared to control

FIGURE 4.2

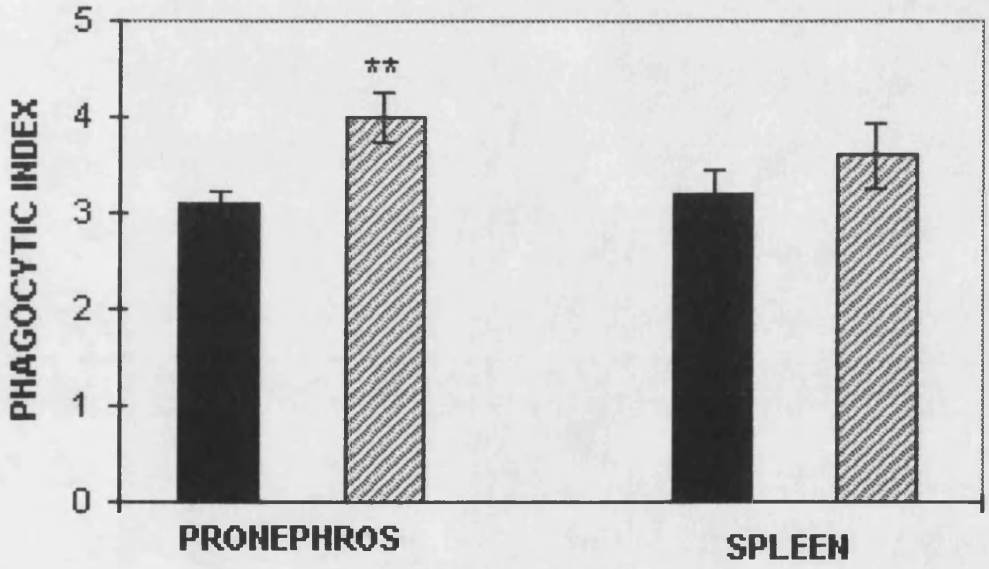


Figure 4.2. The effect of one week post-stress recovery period on macrophage phagocytic index in pronephros and spleen (Expt. C). Control represented by solid bars and recovery represented by single-hatched.

n=5; ** $P<0.01$ compared to control.

4. 4. DISCUSSION

One of the original aims of the present study was to establish the time period required to restore macrophage phagocytic activity after acute stress. The results from this study clearly showed that macrophage phagocytic activity was significantly depressed within 3h of stress and further that it remained depressed for 24 hr. However, when fish were allowed to recover for a week, their phagocytic activity was completely recovered and remained the same thereafter. These findings may have field or aquacultural significance, which indicate that fish should not used for experiments at least for one or two weeks, after they are handled or stressed.

Surprisingly, after six days of recovery period, macrophages from the pronephros often displayed a raised phagocytic activity. This enhancement was significant in three experiments and tended to show an increase in another experiment. The condition of fish and the season in which experiments were carried out may have an influence on the results.

In mammals, various stresses have been shown to alter the immune responses. For instance, T-cell mediated immune responses, such as contact sensitivity reaction to 2,4-dinitro-1-flurobenzene (DNFB) was enhanced within 24-72 hr of 2.5hr of immobilization, cold and heat stress in mice (Blecha et al., 1982) whereas, delayed type hypersensitivity (DTH) to SRBC was enhanced after temperature and cold stress but, suppressed by immobilization stress.

Similarly, even more severe stress, consisting of 14 hr per day restraint for 0, 11, 22, or 33 days, enhanced the cytotoxic response of splenic NK cell against YAC-1 target, and enhanced lymphocyte proliferative response to Con.A and LPS after all time periods (Jain and Stevenson 1991). These responses were quite rapid in other situations: thus, human NK cell activity was significantly increased within 5 min of exercise (Targan et al., 1981; Edward et al., 1984), whereas there was no detectable change in the proliferative response of splenic lymphocytes to Con.A (Targan et al., 1981; Wood et al., 1993) or PHA or LPS (Wood et al., 1993). In another study, at the end of 14 days of daily cold water stress, mice showed enhanced spleen cell mitogenesis to LPS (Aastrad et al., 1991). Similarly, rat splenocyte proliferation in response to Keyhole Limpet Haemocynin (KLH) was significantly increased after one day of footshock stress (Wood et al., 1993), or in response to LPS after 39 days of noise stress (Mojan and Collector 1977). A study by Jessop et al., (1987) has demonstrated that 5 weeks of isolation, plus daily consumption restricted to 30 min, also increased splenic lymphocyte response to PHA this was further increased by 2-3 fold if rats were exposed to this treatment for 12 weeks.

Thus, from the above results it was observed that cellular immune responses can be either increased or decreased by stress. The direction of response is mainly dependent upon the type of immune response and the nature, magnitude, and duration of stress.

The humoral immune responses, may similarly show enhancement by stress. Thus, the anti-sRBC IgM plaque-forming cells in spleen was enhanced within 2hr after brief physical stimuli to normal mice (Fujiwara and Orita 1987), whereas anti-KLH IgG levels

were enhanced on day 0 or 1 in rats exposed to brief footshock electric footshock (Wood et al., 1993).

This stress-induced immune enhancement was not only restricted to cellular and humoral responses of the acquired immune system, but was shown to increase innate immune responses of macrophages such as their phagocytosis and their enzyme contents. Thus, exposure of peritoneal and connective-tissue derived macrophages in mice and humans to a single exhaustive endurance running test increased the phagocytic activity and enzyme content of these phagocytes (Fehr et al., 1988; 1989).

In most of the above cases, stress has been shown to increase immune responses. But, if stress is for short duration, it depresses the immune functions. For instance, Okimura et al., (1986) and Aastrad et al., (1991) observed a diminution in lymphocyte proliferation in response to Con.A and LPS, after they subjected mice to immobilization stress for 12hr daily for 2 days or brief cold stress for one day.

The physiological mechanism whereby stress may facilitate or suppress cell-mediated immunity in mammals and fish is unknown. Stress causes the characteristic alterations in neuroendocrine components, as well as eliciting typical changes in the number of thymus and peripheral blood leukocytes (Riley, 1981). So far as the cellular and humoral immune responses are concerned, the immuno-enhancement following some stresses may be attributable to the activation of the sympathetic nervous system and catecholamine release. Thus, injection of adrenaline enhanced the response of specific helper T lymphocytes when they were challenged with SRBC (Fujiwara and Orita 1987) or the

increase level of the cyclic nucleotide, guanosine 3-5-monophosphate, when lymphocytes are challenged with a mitogen (Mojan and Collector 1977). However, it has also been shown that the activities of immune cells may be enhanced by other neurohormones and cytokines such as growth hormone or interferons etc.

Increased phagocytic activity of the macrophage population seen in response to stress in the pronephros may be due either to the recruitment of a new and active population of macrophages/monocytes or to the elevated activity of individual macrophages present at the time of stress.

The regulation of immune system by many hormones/neurohormones have been known. Thus, immunoregulatory properties of GH hormone have been shown by many authors. For instance, augumentation of cytolytic T cell activity (Snow et al., 1981), NK cells (Kiess et al., 1986) and delayed type of hypersensitivity T cells (Nagy et al., 1985) as well as antibody synthesis in response to T dependent antigens (Nagy et al., 1985) have been demonstrated.

The only line of evidence in fish revealed that GH given 5 days earlier, increases non-specific cytotoxic activity of leukocytes from chum salmon pronephros, spleen and blood when these cells were tested *in vitro* with tumor cells (Kajita et al., 1992). Alternatively, trout express prolactin receptors (Narnaware Y.K.; Unpublished) and prolactin will enhance immune system (Viseli et al., 1991).

In summary, this study shows that macrophage phagocytic activity recovers within a week and unlike in mammals, a short, acute stress can enhance phagocytic activity of macrophages suggesting that environmental stressors not only depress immune responsiveness but can also enhance it.

CHAPTER 5

THE EFFECT OF HORMONES AND NEUROTRANSMITTERS ON MACROPHAGE IMMUNE RESPONSE IN TROUT.

5.1. INTRODUCTION

The regulation of the immune system by the neuroendocrine system is now well established in mammals, mediated in part through the autonomic innervation of lymphoid organs (Williams et al., 1981; Felten et al., 1987; Wan et al., 1993). These authors showed that surgical or chemical lesioning of the nerve supply alters immune capacity, providing evidence for its immunoregulatory roles. These neuroendocrine-induced responses are mediated through specific, high affinity receptors which are present on immune cells. It is now accepted that the immune and nervous systems form a totally integrated circuit by virtue of sharing a common set of hormones and receptors.

As in mammals, the presence of functional adrenergic receptors (Nilsson and Grove 1974) and innervation of lymphoid organs in fish is well established. Chemical denervation results in enhanced plaque-forming cells in immunized fish (Flory, 1989) and metabolic activity of fish leukocytes is influenced by adrenergic and cholinergic receptor agonists (Flory and Bayne 1991). Evidence that products of the peripheral sympathetic nervous system of teleost can influence the antibody response in splenic lymphocytes and the respiratory burst of fish leukocytes have been provided (Flory,

1988; Flory and Bayne 1991a,b), strongly suggesting that the autonomic nervous system plays an important role in the regulation of teleost immunity. However, there is a paucity of information concerning the regulation of macrophage phagocytic function by adrenergic and cholinergic agents *in vivo* and *in vitro.*

In higher animals, stress-induced increase in glucocorticoids and catecholamines have been shown to affect many immune functions. As far as the phagocytic cells are concerned, glucocorticoids have been shown to decrease the number of monocytes/macrophages and neutrophils at the site of inflammation (Thompson and van Furth 1970) depress the killing activity of these cells by reducing lysosomal hydrolyses such as, elastase and collagenase (Werb et al., 1978), suppress nitric oxide synthesis (Cunha et al., 1993), reduce phagocytosis (Thompson and van Furth 1970; Wolff, 1981) and modulate respiratory burst activity (Nielson, 1987). Pharmacological concentration of glucocorticoids rendered the normal macrophages unresponsive to lymphokines (Masur et al., 1982) and decreased their bactericidal capacity (Van Zwet et al., 1971).

Catecholamines have also been shown to modulate many other immune responses including antibody production (Fujiwara and Orita 1985; Madden et al., 1994a,b), cytotoxicity of natural killer cell (Tonneson et al., 1984), and the mitogenic responses of lymphocytes ((Koff and Dunegan 1985). However, glucocorticoids and catecholamines are not always immunosuppressive and occasionally have been found to increase immune responses such as chemotaxis, adherence and phagocytosis of neutrophils (Ortega et al., 1993), phagocytosis in human monocytes (Kay and Czop

1994), plaque forming cells (Fujiwara and Orita 1987; Livnat et al., 1987) and natural killer cell activity (Tonneson et al., 1984).

In fish, most studies on the effect of glucocorticoids have been directed towards the humoral immune response such as a decrease in lymphocyte proliferation (Grimm 1985; Maule et al., 1987; 1989), and depression of antibody production (Anderson et al., 1982; Wechesler et al., 1986). Glucocorticoids also increase susceptibility to fungal and bacterial infection (Pickering and Duston 1983; Maule et al., 1989) and reduce the number of circulating lymphocytes in the blood (Pickering et al., 1987; 1992). The few studies on the effect of glucocorticoids on macrophages include the observation that cortisol reduces the number of monocytes/macrophages and neutrophils at the site of inflammation (MacArthur et al., 1984).

Catecholamines have been shown to modulate other components of non-specific immune response such as metabolic activity, including the respiratory burst (Bayne and Levy 1991a,b). However, the effect of catecholamines on macrophage phagocytic activity has not yet investigated.

In the previous chapter, it was shown that stress, which is known to raise plasma cortisol and epinephrine and to stimulate nor-adrenaline release at nerve terminals, results in a depressed phagocytic index of pronephros and spleen macrophages.
To expand this knowledge of neuroendocrine regulation of macrophage phagocytic activity by cortisol and catecholamines, the effect of both cortisol and catecholamines on macrophage phagocytosis has been studied *in vivo* and *in vitro*

5.2. MATERIALS AND METHODS

5.2.1. *In vivo* and *in vitro* treatment with cortisol.

For *in vivo* administration of cortisol (Hydrocortisone, Sigma) it first dissolved in a small volume of ethanol and then diluted in Leibovitz medium (L-15 medium, Sigma). Fish were anaesthetised and injected intraperitoneally with cortisol (50 μg/0.5 ml) or 0.8 % saline as a control. They were returned to their home tanks for 3h. A further group of control fish were left undisturbed throughout experiment.

Cells from pronephric and splenic suspensions were distributed onto several separate glass slides and left 1.5h to adhere. Slides, with their adherent macrophages, were then incubated for 3h in medium alone or with medium containing cortisol (80 ng/ml) before the addition of yeast cells.

5.2.2. *In vivo* and *in vitro* treatment with α- and β adrenergic agent agonists and antagonist

Nor-adrenaline (Bitartrate salt, Sigma) or phentolamine (Sigma) was prepared immediately before use in 0.8 % saline and kept on ice. Fish were injected intraperitoneally, without anaesthesia, with 0.5 ml saline containing 0.25 mg or 2.5 mg nor-adrenaline, or 5mg phentolamine (Sigma), or saline alone. Immediately after this, fish were returned to their home tanks and left for 3h. After 3h, all groups were anaesthetised, blood was collected and pronephros and spleen cell suspension was

prepared as described previously (chapter 2). Subsequent presentation of yeast cells and staining was done as described in chapter 2.

For the *in vitro* study, cells from pronephric and splenic suspensions were each distributed over 4 slides. The adherent macrophages were incubated for 3h or 6h in medium containing the adrenergic agonists, phenylephrine (10^{-5}M) isoprenaline at (10^{-5}M) or a combination of nor-epinephrine and cortisol.

5. 3. RESULTS

5.3.1. *In vivo* and *in vitro* effect of glucocorticoid on macrophage phagocytosis.

Intraperitoneal (i.p.) injections of either saline or 50 μg cortisol were administered and the response of macrophages was assessed 3h after the injections. As in chapter 3, saline injection alone depressed the % phagocytosis and phagocytic index of both the pronephric and splenic macrophages (Table 5.1; Fig. 5.1). Plasma cortisol values were raised to 20 ng/ml by this handling stress. The i.p. injection of 50μg cortisol/200 g fish resulted in a greater rise in plasma cortisol to give values of between 60-74 ng/ml (Table 5.1 A, B). However, this did not further depress macrophage activity. On the contrary, neither the % phagocytosis and P.I. in these fish responded to the injection stress, but now resembled the uninjected control.

To assess the effect of cortisol *in vitro*, macrophages were incubated in medium alone (control) or medium containing 80 ng/ml cortisol for 3h. The results presented in table 5.2A show that neither the percentage of phagocytosis nor P.I. of cells from either tissues were significantly depressed within a 3h incubation period. This was confirmed in a second experiment (not shown). However, 6h incubation of macrophages with cortisol now significantly depressed the phagocytic activity of pronephric but not splenic macrophages (Table 5. 2B).

Table 5.1.

***In vivo* effect of cortisol on macrophage phagocytosis.**

TREATMENTS	PRONEPHROS		SPLEEN		CORTISOL
	% PHAGO.	P.I.	% PHAGO.	P.I.	(ng/ml)
A. UNINJECT.	93±0.9	3.6±0.08	97±1.1	4.1±0.22	6±1
SALINE	78±3.2**	2.3±0.11**	82±2.7**	2.6±0.09**	20±3**
CORTISOL	89±1.9$	3.2±0.10*$$	94±2.3$	3.8±0.15	60±2.7**$
B. UNINJECT.	96±1.7	3.4±0.10	------	----	1±0.13
SALINE	81±3.5*	2.3±0.16**	-------	----	17±11.9**
CORTISOL	97±0.6$	3.4±0.11$$	--------	------	74±11.9**$$

Table 5.1. Fish were lightly anaesthetised and injected i.p. with 0.5 ml saline (0.8% NaCl) or 50 µg/0.5 ml cortisol and killed 3h after injection together with an uninjected control. 200 macrophages per fish were assessed to determine % of phagocytosis and phagocytic index.

* P<0.05 ** P<0.001 compared to uninjected
$ P<0.05 $$ P<0.01 compared to saline injected.

FIGURE 5.1

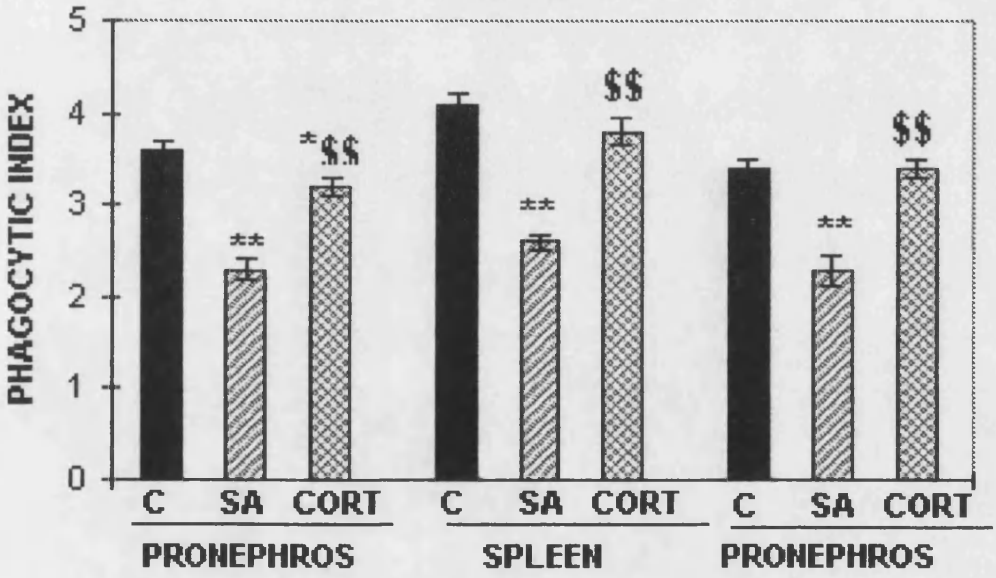


Figure 5.1. The effect of 3h *in vivo* administration of cortisol at a concentration of 50 μg/0.5ml of saline on pronephric and splenic (Expt. 1) and only pronephric (Expt. 2) macrophage phagocytosis. Control (C)- solid bars; saline injection (SA)- single hatched bars; cortisol injected (CORT)- crossed-hatched bars.

* P<0.05 ** P<0.001 compared to uninjected
$ P<0.05 $$ P<0.01 compared to injected

Table 5.2.

The effect of *in vitro* cortisol on macrophage phagocytosis

TREATMENT	PRONEPHROS		SPLEEN	
	% PHAGO.	P.I.	% PHAGO.	P.I.
		(5.2 A)		
3 HOURS				
CONTROL	88±2.94	2.8±0.19	85±4.6	2.9±0.14
CORTISOL	87±3.72	2.8±0.26	82±7.9	2.6±0.25
		(5.2 B)		
6 HOURS				
CONTROL	99±0.19	5.0±0.20	100±0.0	5.0±0.16
CORTISOL	94±1.35**	3.7±0.06**	100±0.0	5.0±0.18

Table 5.2: Macrophages were incubated with or without medium containing 80 ng/ml cortisol for 3h or 100 ng/ml for 6h. Percentage of phagocytosis (% phago.) and phagocytic index (P.I.) were determined by observing 200 macrophages from each fish. (n=4 fish per treatment).

** $P<0.01$ Compared to control.

5.3.2. *In vivo* and *in vitro* effects of catecholamines on macrophage phagocytosis.

The results from above experiments showed that cortisol applied to macrophages under either *in vivo* or *in vitro* conditions did not directly modulate their phagocytic activity within 3h. Whether the depressive effect of handling was attributable to catecholamines was assessed in further experiments.

Fish were injected with either nor-adrenaline or the α- receptor antagonist, phentolamine. In the first experiment (Table 5.3A) saline injection, exceptionally, had no effect but nor-adrenaline significantly lowered the P.I. of both pronephric and splenic macrophages compared to injected controls (Fig. 5.2A). Fish injected with phentolamine had a phagocytic activity equal to that of control fish. Additionally, phentolamine induced engorgement of the spleen with blood (148 % weight increase i.e. control 2.07±0.34 and phentolamine 3.05±0.39 compared to saline).

In a second experiment, both saline and nor-adrenaline injections significantly depressed phagocytic activity, but in this case nor-epinephrine was no more suppressive than handling stress alone (Fig. 5.2B). Again the α- adrenergic blocker prevented this depression. Indeed, phagocytic activity of spleen macrophages was significantly raised compared with both uninjected and injected controls (Table 5.3B). Again, an enlargement of spleen size, compared to control was obvious (see above). Despite its ability to prevent the macrophage response to stress, phentolamine did not prevent the rise in plasma cortisol levels. Surprisingly, the cortisol response to

Table 5.3.

***In vivo* effect of nor-adrenaline or an adrenergic antagonist on macrophage phagocytosis.**

TREATMENT	PRONEPHROS		SPLEEN		CORTISOL
	% PHAGO.	P. I.	% PHAGO	P. I.	(ng/ml)
		(Table 5.3A)			
UNINJECT.	90±3.1	3.1±0.22	95±1.0	3.9±0.28	2±0.9
SALINE	92±2.2	3.3±0.1 2	95±2.0	4.0±0.18	2±0.9
NOR-ADR. (0.25mg)	91±1.1	3.0±0.07&	82±4.5*&	3.0±0.33&	49±7.0**&&
PHENTO.	90+3.9	3.2±0.13	94±3.5	4.2±0.20$	31±8.7**&&
		(Table 5.3B)			
UNINJECT.	97±0.68	4.1±0.06	99±0.6	4.4±0.20	8±3.3
SALINE	81±5.0**	2.5±0.25**	74±3.3**	2.7±0.20**	28±6.7**
NOR-ADR. (2.5mg)	80±2.3**	2.4±0.13**	88±2.4**	3.0±0.07**	6±1.5
PHENTO.	95+2.6&$	3.7±0.13$$&&	99±0.3$	5.0±0.08*$$&&	43±6.8$**

Table 5.3A. Fish were anaesthetised and injected i.p. with saline (0.8%) NaCl), nor-adrenaline (0.25mg/0.5 ml) or phentolamine (5 mg/0.5 ml) (n=5).

Table 5.3B. Fish were injected i.p. without anaesthesia with nor-adrenaline (2.5 mg/0.5 ml) or phentolamine (5 mg/0.5 ml). In both experiments fish were killed 3h after injections. (n=5-6)

* P<0.05	** P<0.01 compared to uninjected
& P<0.01	&& P<0.001 compared to saline injected
$ P<0.01	$$ P<0.001 compared to nor-epinephrine

FIGURE 5.2A

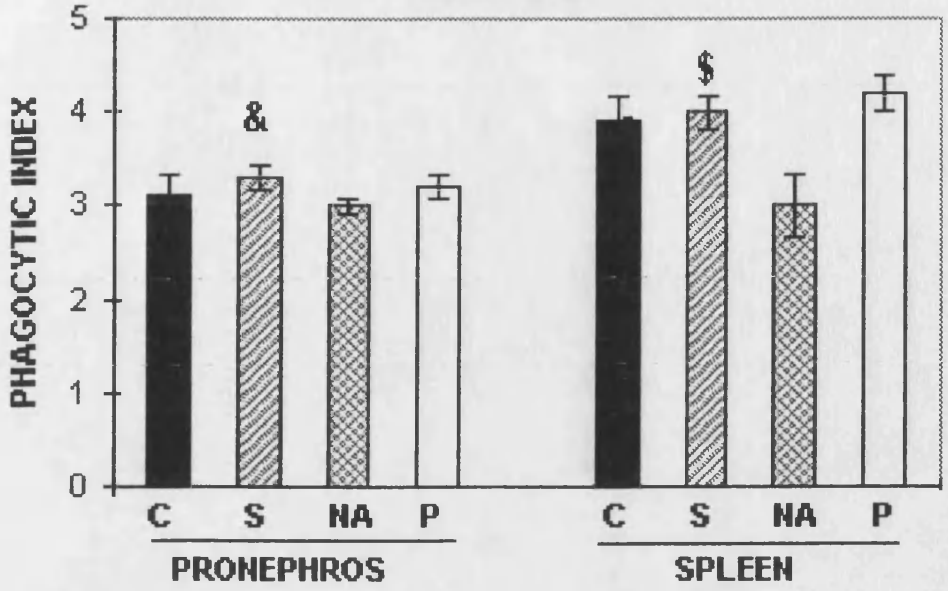


FIGURE 5.2B

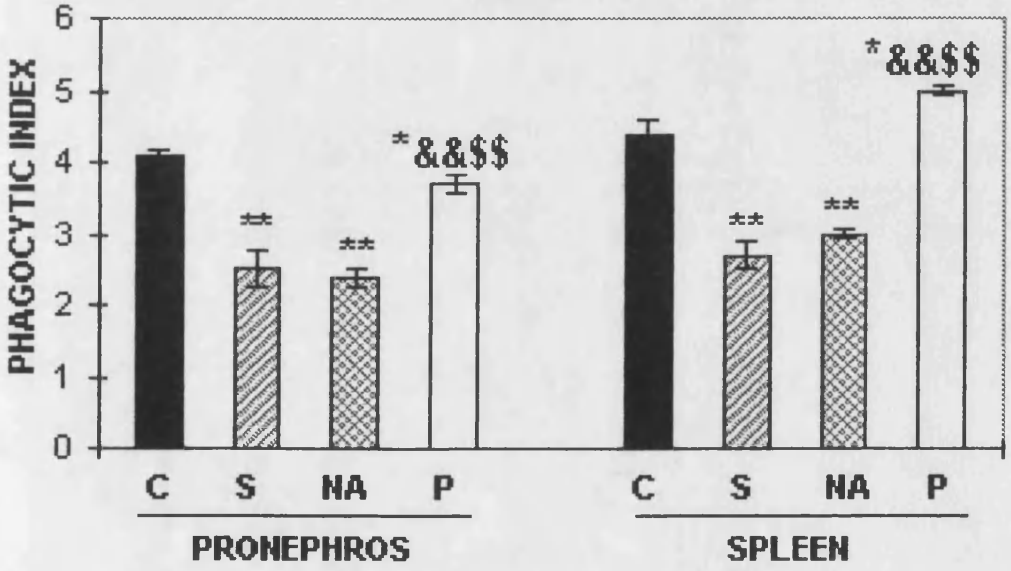


Figure 5.2A&B. The effect of 3h *in vivo* treatment with adrenergic agonist (Nor-adrenaline) at 0.25mg/0.5ml of saline (Table 3A) or 2.5mg/0.5ml saline (Table 3B) and the adrenergic antagonist, phentolamine at 5mg/0.5ml on both pronephric and splenic macrophage phagocytosis. Control (C) solid bars; saline injected (S) -single hatched bars; nor-adrenaline (NA)-cross-hatched bars and phentolamine (P)-empty bars.

* P<0.05	** P<0.01 compared to uninjected
& P<0.01	&& P<0.001 compared to injected
$ P<0.01	$$ P<0.001 compared to nor-adrenaline

nor-epinephrine was variable - raised after a low dose of nor-epinephrine, but remaining low after a high dose.

To confirm the effect of catecholamines on macrophage phagocytic activity, the effect of α- and β- adrenergic agent agonists were tested *in vitro*. In two separate experiments, the α-adrenergic agonist, phenylephrine (10^{-5}M) caused a significant depression in P.I. within 3h and 4.5h (Table 5.4A & B; Fig. 5.3A & B). although the % phagocytosis was virtually unaffected by the treatment. The β-adrenergic agonist, isoprenaline also depressed the phagocytic activity of pronephric macrophages at 10^{-5}M in both experiments but spleen macrophages responded to this β- agonist in only one of the two cases (Fig. 5.3A).

Table 5.4.

The effect of α- and β- adrenergic agonists on macrophage phagocytosis *in vitro.*

TREATMENT	PRONEPHROS		SPLEEN	
	% PHAGO.	P. I.	% PHAGO.	P. I.
A. CONTROL	97±0.7	4.2±0.16	98±0.4	4.0±0.06
PHENYL (α-)	96±1.0	3.2±0.11**	96±1.6	3.4±0.15**
ISOPR (β-)	98±0.5	3.6±0.05*	97±1.1	3.9±0.05$
B. CONTROL	97±0.6	4.5±0.12	98±0.5	4.9±0.15
PHENYL (α-)	94±2.3	3.9±0.11**	96±1.1	3.9±0.10**
ISOPR (β-)	94±1.5	3.7±0.22*	96±0.7	4.1±0.09**

Table 5.4. Macrophages from both tissues were incubated with or without phenylephrine and isoprenaline at 10^{-5}M for 4.5 h (Table 4A) and 3h (Table 4B) and macrophage phagocytic activity was determined from 200 macrophages/fish/tissue. (n=5 fish per treatment).

* P<0.05 ** P<0.01 compared to control
$ P<0.02 compared to phenylephrine

FIGURE 5.3A

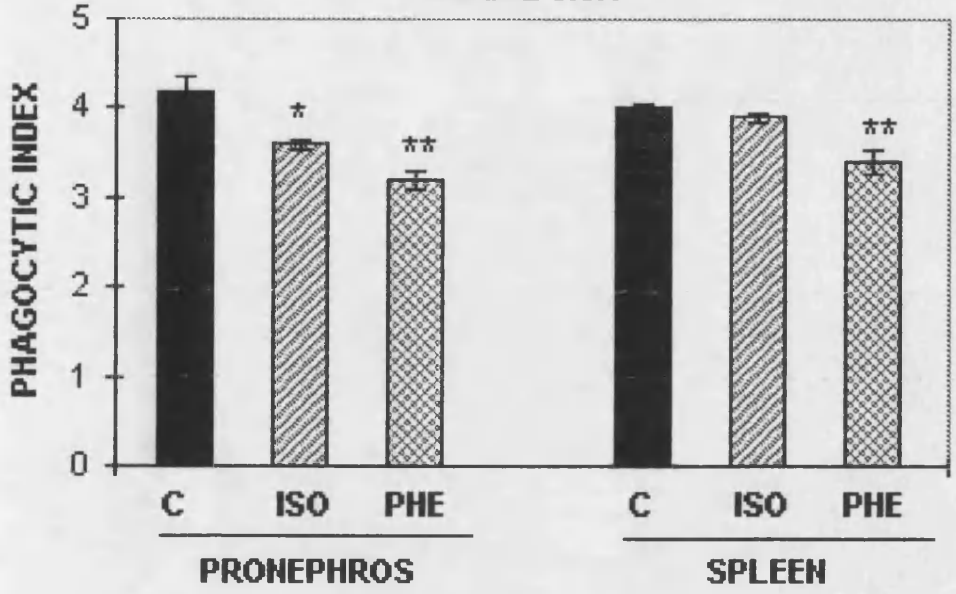


FIGURE 5.3B

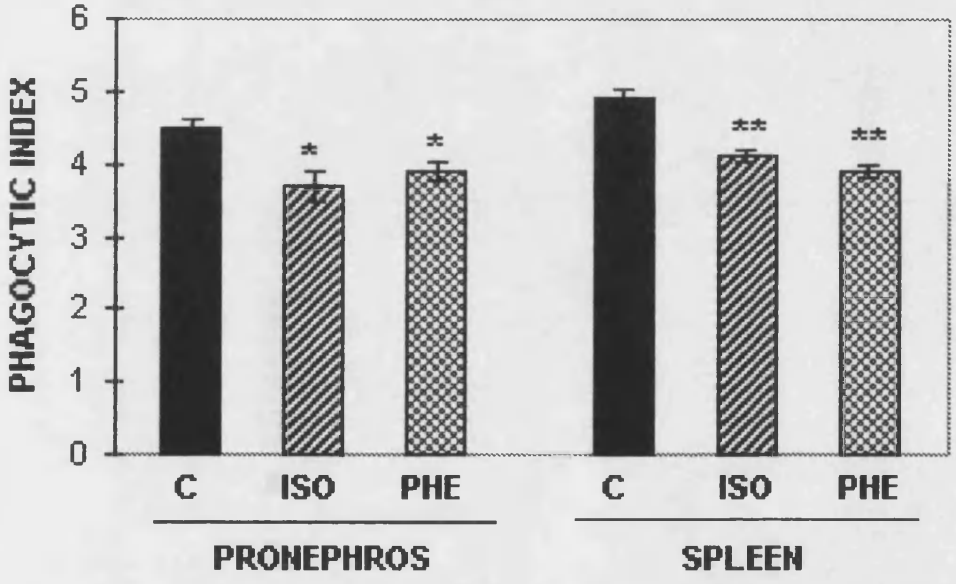


Figure 5.3A&B. The effect of 3h (Fig. 5.3A) or 4.5h (Fig. 5.3B) *in vivo* treatment with α- and β- adrenergic agents, isoprenaline (ISO) and phenylephrine (PHE), both at 10^{-5} M, on pronephric and splenic macrophages. Control (C)- solid bars; isoprenaline- single hatched; phenylephrine- crossed-hatched.

n=5 *P<0.02 P<0.002 compared to control.

5.4. DISCUSSION

The results show that both catecholamines and cortisol can exert depressive effect on phagocytic activity of macrophages, but the conclusions are less straight forward than would first appear.

In vitro, both α- and β- adrenergic agonists decreased phagocytic index. The measurements of respiratory burst following catecholamine administration in previous studies gave contrasting results regarding macrophage activity however. Thus, in fish, α- adrenergic receptor agonist phenylephrine (PHE) increased the respiratory burst activity, measured by chemiluminiscence, whereas isoproterenol (ISO), a β-adrenergic receptor agonist decreased it. (Bayne and Levy 1991 a,b). However, when monitored by the reduction of cytochrome C, ISO increased responses whereas PE decreased them. Rapid and potent catecholamine effects on other non-specific responses have been observed within a few minutes. Decreases in superoxide anion and chemiluminiscence response of head-kidney macrophages were evident within 4-10 min of phenylephrine treatment in fish (Bayne and Levy 1991 a,b; Flory and Bayne 1991) and in mammals, PMN activation was seen within 7 minutes in response to isoproterenol (Nielson, 1987). Other rapid responses to catecholamines include the decrease in proliferative response of rainbow trout leukocytes to mitogens after 10 min treatment (Flory and Bayne 1991) or the decreased blastogenic response of human lymphocytes to phytohemagglutinin (PHA) after only 15 min of epinephrine administration (Crary et al., 1983).

Decreased phagocytic activity in response to nor-adrenaline administration *in vivo* was greater than that seen *in vitro* (approx. 25-30% reduction compared to 15% reduction *in vitro*). Even very high doses, nor-adrenaline had no greater effect than handling stress. It is possible that the amount of adrenaline released by stress already causes maximal reduction in PI. The decrease in phagocytic activity caused by stress was effectively blocked by phentolamine indicating that the depressive agent is indeed a catecholamine. Taken together, these *in vitro* and *in vivo* results confirm that catecholamines exert a rapid effect on macrophage phagocytic activity and that the response is mediated through α- adrenergic receptors present on macrophages and possibly on the vasculature. The phentolamine-treated group of fish bled readily and the spleen mass was significantly increased as compared to saline injection. This emphasizes the role of adrenergic agents on blood flow.

In a previous chapter it was suggested that the decline in PI and % phagocytosis might be attributed to the removal of active phagocytes from the spleen and pronephros. If this were to be caused by increased adrenaline/nor-adrenaline, influencing the flow of blood through these tissues, then the increased PI following phentolamine would be explained. It might also explain the greater reduction in PI observed after *in vivo* injection of nor-adrenaline than that seen *in vitro*. Nevertheless, the fact that adrenergic agents can influence phagocytosis when they are applied *in vitro*, when considerations of blood flow no longer apply, indicate that catecholamines also exert a direct effect on the macrophages.

Glucocorticoids are hormones used as anti-inflammatory and immuno-suppressive drugs (Russo-Marie, 1992). In mammals, they have been reported to interfere with a variety of both intra and extracellular activities of immune cells. They diminished monocyte chemotaxis (Rinehart et al., 1974), bactericidal capacity (van Zwet et al., 1975) and inhibit macrophage proteinase secretion (Werb, 1978). Glucocorticoids have also been shown to decrease murine peritoneal macrophage phagocytosis which is depressed after 6 days incubation with dexamethasone (Grasso et al., 1981) and to reduce their peritoneal abundance within 6 hrs of administration (Thompson and van Furth 1969). In rats macrophage phagocytic activity was depressed after several weeks of treatement (Wolff, 1981), and this increases the susceptibility of the animals to infection (Schaffner, 1985).

The results from the previous chapter suggested that long term corticosteroid administration would depress PI. The present *in vitro* work shows that cortisol takes more than 3hr to depress phagocytic activity, but some depression is seen after 6hr. This suggests that, in fish, cortisol has a slow onset effect on macrophage phagocytic activity. The results from other studies in higher animals, provide evidence that corticosteroids usually depress phagocytosis, but there is an apparent lack of time response studies.

Most previous studies that have been able to demonstrate an immediate and direct effect on one or other aspects of macrophage activity, have usually used supra-physiological concentrations of steroids, in the range of 1-100 μg/ml (Balow and Rosenthal 1973; van Zwet et al., 1975; Masur et al., 1982). While other authors have

shown that lower concentration (1-100nM) of dexamethasone can influence mouse macrophage activity (Werb, 1978), the response was dose and time related and affected only certain aspects of macrophage activity. Thus, depression in plasminogen activator secretion was detectable 2h after the addition of 100 nM (36 ng/ml) but not 1 nM dexamethasone. Other macrophage enzymes, such as elastase, were less sensitive to this steroid treatment suggesting that cortisol acts differently on different components of macrophage activity.

Cortisol affects not only on macrophages but also other immune cells. For example, NK cell activity was decreased within 6 hr incubation with dexamethasone (Holbrook et al., 1983) but was unchanged even after 24 hr of incubation with hydrocortisone succinate in another study (Onsrud and Thorsby 1981). This slow response to glucocorticoids was observed in other cellular responses such as antibody-dependent cell cytotoxicity (ADCC) and cellular cytotoxicity (Parillo and Fauci 1978) or the secretion of antibodies (Cupps et al., 1984) which was modulated only after 4-6 hr of steroid administration in humans (Parillo and Fauci 1978) or more than week in rats (Wolff, 1981).

In fish, implants of cortisol increase the susceptibility of trout to infection (Pickering and Duston 1983) and reduce the production of antibody secreting cells (Kaattari and Tripp 1987; Tripp et al., 1987; Maule et al. 1987). However, cortisol has not consistently been found to depress all aspects of immunological activity- for instance, plaice (*Pleuronectes platessa*) injected with 3mg/300g body weight dexamethasone for 3 days showed no change in the rate of *in vivo* antigen clearance or organ uptake

of antigen (MacArthur and Fletcher 1985), but its prolonged administration in fish (Narnaware et al., 1994) or in mammals (Grasso et al., 1981), even at a small dose for 6 days or more days produced suppression in phagocytic activity, confirming its potency on this immunologic feature.

The *in vivo* effect of cortisol was surprising. *In vivo,* cortisol effectively inhibited the suppression in PI caused by saline injection which, it is suggested, causes release of nor-adrenaline and adrenaline. This suggests that cortisol may antagonize the release of catecholamines. In mammals, adrenalectomy increased urinary secretion of catecholamines, and cortisol reversed this increase (Parvez and Parvez 1974). Whereas adrenalectomy or hypophysectomy increased turnover of nor-adrenaline in the rat heart, administration of ACTH or glucocorticoid attenuates the increase in cardiac nor-adrenaline turnover (Axelford, 1977; Dailey and Westfall 1978). Recent evidence in rat by Kvetnansky et al., (1993) showed that adrenalectomy or immobilization stress both cause an increase in nor-adrenaline release and 2 hrs pretreatment with exogenous cortisol depressed the increase seen 20 min of immobilization stress (Brown and Fisher 1986; Kvetnansky et al., 1993), suggesting that endogenous cortisol can restrain catecholamine release via feedback relay on CRF.

It is worth considering how a slow effect of cortisol on macrophages might occur. This inhibitory effect of cortisol on macrophages seems to be an indirect one, stimulating the synthesis of other inhibitory proteins such as the lipocortins. For instance, the inhibitory action of the glucocorticoids on the activated HPA axis,

observed *in vivo* and *in vitro,* were mimicked by lipocortin-1 (LC-1) and reversed by neutralizing anti-LC1 (Taylor et al., 1993; Buckingham et al., 1994). It is because steroids usually act genomically, stimulating the transcription and translation of another product, that their effects take time to become apparent. However, it is also possible that under *in vivo* conditions when cortisol prevented the decline in PI normally caused by handling stress, cortisol may have had a fast-feed back effect, and may have inhibited the release of other hormones or neurotransmitters (i.e. the catecholamaines), preventing their action on macrophages.

Fast-feedback by corticosteroids is a relatively recently studied topic. It presumes that the steroid acts on membrane receptors rather than by the much slower genomic route. Evidence for the existence of membrane receptors eg. for the steroid aldosterone, has been advanced recently (Christ et al., 1994). Evidence for the operation of glucocorticoid fast-feedback comes from studies on the rat. Thus, Yates et al., (1961) demonstrated that injection of corticosteroids in rats inhibited the activation of the HPA axis in response to histamine administration if the injection of exogenous steroid preceded histamine administration by 5 min or less, but not if the corticosterone was injected 15 min before or 2 min after the administration of histamine. Similarly, infusion of corticosterone 10 min prior to stress decreased the stress-induced increase of HPA axis activity (Jones et al., 1972). Thus, it was proposed that there was a rapid inhibitory effect of corticosterone that occurs while plasma concentrations of the hormone were increasing. Jones et al., (1972) and Abe and Critchlow (1977) suggested that rapidly occurring component of corticosteroid feed-back was rate sensitive i.e. responded to rapidly rising titres of steroid values of

1-2 μg/100 ml/min. Others have also shown that administration of adrenal cortical extract or cortisol, before but not after, stimulation of the pituitary-adrenal axis inhibited the response to the stress (Hodges and Jones 1964). The evidence to date suggests that, after stimulation, the brain-pituitary-adrenal axis operates in a closed-loop manner, fast-feedback operating immediately after steroid administration and the slow feedback system coming into action 1-2 hr later, after plasma corticosteroid concentrations have decreased (Keller-Wood and Dallman 1984).

Catecholamines are known to be released very rapidly upon stress, and to have immediate and enduring effects on macrophages and other leukocytes. Thus, one must presumes that if exogenous cortisol protects the macrophages from the depressive effects of catecholamines, it must act very rapidly to prevent catecholamine release i.e by a fast-feedback route. The time scale during which cortisol has a fast-feed back effect in mammals after stress makes it problematical whether such a feedback could be operating here. In mammals, an immediate or 2 min period after the stress is available for the fast-feedback action. It is uncertain whether cortisol, injected i.p. could reach the brain fast enough to depress CRF release, and CNS activation. Alternatively, cortisol may reach the spleen rapidly and perhaps prevent local sympathetic nerve depolarization at this site. Depolarization of hypothalamic cell membranes with K^+ prevented the corticosterone inhibition of CRF release in mammals (Jones and Hillhouse 1976; Jones et al., 1977). Further studies are clearly needed.

In summary, this chapter suggests the depressive effect of stress on phagocytosis could be due to the release and action of catecholamines and cortisol. The effects of catecholamines are rapid and are two-fold one effect may due to an influence on blood flow, washing out active macrophages from the spleen and pronephros, so that the residual macrophages show an apparently reduced phagocytic activity. The *in vitro* study indicates, however, that catecholamines may also act on adrenergic receptors on the macrophages and have a direct depressive effect on the phagocytosis. Cortisol also seemed to have a two-fold effect on the phagocytes, when administered for more than 3hr, it had an apparently direct depressive effect on macrophage phagocytosis, presumably by the classical slow-feedback pathway involving lipocortin production. By contrast, when administered by injection *in vivo*, it counteracted the effects of stress. It is postulated this might be via fast-feedback effect, inhibiting either the release or action of catecholamines.

CHAPTER 6

EFFECT OF MILD AND INTENSE ACUTE STRESS, CORTICOSTEROID AND CATECHOLAMINES ON CIRCULATORY LEUKOCYTE NUMBERS IN TROUT.

6. 1. INTRODUCTION

Intensive aquaculture of fish often necessitates the use of various handling and transport procedures which frequently causes stress resulting in detrimental consequences such as morbidity or mortality. Stresses associated with aquaculture practices often reduce the circulating lymphocytes in fish. Thus, the number of lymphocytes has been shown to decrease in response to various stresses such as, handling and crowding in coho salmon, *Oncorhynchus kisutch* (Wedemeyer 1976) and rainbow trout, *Oncorhynchus mykiss* (Pickering et al., 1982; Angelidis et al., 1987; Barton and Iwama 1991), Pike, *Esox lucius* (Soivio and Oikari 1976), handling and capture, in *Fundulus heteroclitus* (Slicher, 1961), cold shock in *F. heteroclitus* (Pickford et al., 1971) and only handling in marine dab, *Limanda limanda* (Pulsford et al., 1994). Similarly, acid stress in freshwater fish, *Channa punctatus* (Dheer et al., 1987) and thermal stress in goldfish, *Carassius auratus* (Dunn et al., 1989) have been shown to reduce lymphocyte numbers in circulation and decrease their responses to mitogens *in vivo* in other species (Ellsaesser and Clem 1986; Maule et al., 1987; 1989; Pickering and Pottinger 1992) and *in vitro* (Miller and Tripp 1982; Tripp et al., 1987; Anderson et al., 1989). Thus lymphocyte numbers commonly decline not only after stress (Pickering et al., 1982) but also in response to pollutants

such as craft mill effluents (McLeay et al., 1973 a, c) and treatment with therapeutants such as malachite green (Hlavek and Bulkely 1980). On the other hand, stress also increases numbers of circulating phagocytes such as neutrophils (Hines and Spira 1973; Ellsaesser and Clem 1986; Lehmann et al., 1987; Dheer et al., 1987; Dunn et al., 1989; Pulsford et al., 1994a) and macrophages (Pulsford et al., 1994a). Their numbers have also been shown to increase in response to contaminated sediments and sewage in laboratory experiments (Secombes et al., 1991; Tahir et al., 1993; Pulsford et al., 1994b)

In fish, the change in leukocyte numbers in response to infection has been conflicting and depends upon the stage of infection. For instance, leukocytes of unspecified cell type were increased in advanced stages of infection in carp *(Cyprinus carp)* infected with *Pseudomonas alcaligenes* and *Aeromonas salmonicida* (Siwicki and Studnika 1987). whereas mirror carp infected with *Ichthyophthiriasis multifillis* showed decline in lymphocyte numbers after 2 days, followed by a return to normal after 16 days (Hines and Spira 1973). In *O. mykiss*, similar, experimentally infected fish (Red mouth disease) also showed a decline in lymphocyte numbers on the third and ninth day of infection (Lehmann et al., 1987). Pickering and Pottinger (1992) similarly, observed mortality of Atlantic salmon *(Salmo salar)* due to bacterial fin rot or furunculosis was associated with decreased lymphocytes numbers in the blood. Infection also increased phagocytes such as neutrophils (Hines and Spira 1973; Lehmann et al., 1987) and macrophages (Lehmann et al., 1973)

In mammals, the leukocyte response to various stresses are variable and conflicting. Intense acute stress such as restraintment in monkeys (MorrowTesch et al., 1993) and

rats (Dhabhar et al., 1994) and injury in human (Miller and Baker 1979) all decrease the circulating lymphocytes and *in vitro* response to mitogens, whereas, some stresses, such as exercise for a short duration have been found to either decrease (Steel et al., 1974) or increase lymphocytes numbers (Edwards et al., 1984; Murray et al., 1992; 1993; Maisel et al., 1994), Although, depressing their response to mitogens (Murray et al., 1992). As in fish, phagocytes have been shown to increase in response to stress (Bermudez et al., 1990; Aastrad et al., 1991) and infection (Gurney et al., 1992; Bodey et al., 1994). During infection, an increased leukocytes count is observed which may be attributable to enhanced lymphocytes (B-cells) (van Kuyl et al., 1994) or T-cells (Verani and Girott 1993) or to neutrophils (Verani and Girott 1993).

Growing evidence in mammals suggests that immune functions are regulated in part by the sympathetic nervous system. Sympathetic nerve endings densely innervate lymphoid tissues such as spleen, lymph node and, thymus, and lymphoid cells have α- adrenergic receptors (Felten et al., 1987). There is also evidence that activation of the sympathetic nervous system influences the distribution of lymphocytes between the tissues and the blood i.e. immunoregulatory cell trafficking (Madden et al., 1994 a,b).

Acute α- adrenergic stimulation will enhance the number of circulatory lymphocytes in humans and rats. This occurs in response to various stresses such as dynamic exercise or psychological stress (Landmann et al., 1984; Maisel et al., 1990 a; Murray et al., 1992; 1993) and certain disease conditions like Systemic Lupus Erythomatous (SLE) (Morimoto et al., 1980) and congestive heart failure (Maisel et al., 1990) or infusion of epinephrine (Crary et al., 1982; van Titis et al., 1990) or isoproterenol (Murray et al.,

1992). However, there is no comparable evidence in fish to show the sympathetic system regulates circulatory lymphocyte numbers.

The aim of the present chapter was to correlate changes in leukocytes- lymphocytes and phagocytes, which occur in response to various stresses to determine if such changes could be useful as an indicator of stress or infection.

Also studied was the question of whether tank colour could influence the number of lymphocytes in response to stress. During adaptation to a pale-coloured background trout secrete the neuropeptide, Melanin-Concentrating-Hormone (MCH) which influences the HPI axis, depressing the steroidal response to stress (Green and Baker 1991). Because MCH is released in fish when they adapt to a pale background, the HPA axis will also respond differently on white and black backgrounds (Gilham and Baker, 1984; Baker et al., 1985; 1986). Moreover, MCH has been shown to be influenced by stress, and its release is enhance under stressful conditions (Green et al., 1991). Whether stress differently influences the lymphocyte number in white and black adapted fish has also been investigated in this chapter.

6.2. MATERIALS AND METHODS

6. 2. 1. Fish.

Rainbow trout *(Oncorhynchus mykiss)* eggs were obtained from a local hatchery and the fry were reared in either white or black tanks for 15 months. At the time of experiments they weighed up to 250g. They were kept on a 18h light:6h dark photoperiod and fed on commercial trout pellets.

For some experiments, trout weighing approximately 250g were obtained from a local fish farm (Alderely Trout Ltd, Wotton-Under-Edge, Glouc.) and acclimated at aquarium conditions on white tank for at least 2 weeks before the start of the experiments.

6. 2. 2. Administration of short and acute stresses.

Fish reared on white and black background were stressed in a number of ways:

a) Holding them in air in a net for 2 min. They were then returned to another tank for a 2h recovery period, after which they were anaesthetised and killed.

b) Fish from either background were transferred to a tank with 1.5 inches of water. They were left in low water for 3 min and immediately transferred to another tank for 4hr or 28hr recovery periods before autopsy.

c) Some white- and black-reared and also farm fish maintained in white tanks were stressed by 1h noise plus a further 2h confinement as described in chapter 3 and killed immediately after the 3h period.

d) Fish were netted and transferred into warm water at 25°C. They were left at this temperature for 4-5 minutes, till they were unconscious and lying upside down. They were then immediately transferred to a tank for 3hr recovery and then killed.

e) Fish were netted out of their home tank into a tank of phenoxyethanol anaesthetic, left for 2 minutes and then returned their home tank to recover.

6. 2. 3. Administration of chronic stress.

Five farm fish were stressed daily for 6 days by 1h noise plus 2h further confinement; they were killed, with control fish, at the end of the 3h period on the last day of stress.
In another experiment, fish were transferred twice daily, for 6 days, to a tank containing one and half inch water and so stressed for 3 minutes. They were killed after the second stress on the sixth day.

6. 2. 4. Treatment with cortisol, adrenergic agonist and antagonist.

Fish were caught in a single scoop, anaesthetised or not, depending upon the experiment and injected i.p. with either cortisol (50μg/0.5 ml saline), nor-adrenaline (2.5mg/0.5 ml saline) or phentolamine (5mg/0.5 ml saline) and killed after 3h of the injection procedure.

6. 2. 5. The effect of infection on leukocytes, hormone values and spleen size.

Trout claimed to be carrying an infection were caught at the fish farm. They were identified on the basis that they were dark in colour, had slightly red bellies and swam apart from the common shoal. Control fish were similarly caught. Both groups were acclimated to aquarium conditions for a week, in separate white tanks, and then killed for tissue inspection.

6. 2. 6. Collection of blood and leukocytes counts.

Approximately 2-3 ml blood was collected from each fish in ice-cold tubes containing 50 µl 6% EDTA as anticoagulant. To count the number of leukocytes, 50, µl in the case of the lymphocyte count, or 100 µl in the case of phagocyte counts, were mixed in Rees-Ecker stain to give 1 ml mixture and were counted using a haemocytometer (Neubauer). The leukocyte counts were expressed as thousands of cells per microlitre.

Plasma cortisol values in these stressed fish was measured by RIA as decribed in chapter 2.

6. 3. RESULTS

The lymphocyte response to a range of different stresses was investigated. Whether examined after 3h or after repeated stress, fish always showed a significant decrease of circulating lymphocytes, 66-79% of control (Tables 6.1 A-D). The control values ranged between 31-26 K/μl and stressed fish had values ranging from 21-13 K/μl. No apparent adaptation to repeated stress was observed in lymphocyte depression in contrast to macrophages, although cortisol levels were lower after repeated than after a single stress (Table 6.2; Fig. 6.1). There was no apparent correlation between plasma cortisol and the percent decrease in lymphocyte numbers ($r = -0.39$). However, fish left to recover for 6 days showed lymphocyte numbers which were no longer significantly different from undisturbed controls (Table 6.1).

To investigate the potential cause of this decline in lymphocyte numbers, fish were injected with either cortisol (50 μg/fish), nor-adrenaline (2.5 mg/fish) or the α-adrenergic antagonist, phentolamine (5mg/fish).

In two separate experiments, cortisol prevented the lymphopenia caused by a saline injection (Table 6.3; Fig. 6.2). Surprisingly, both nor-adrenaline and the α-adrenergic blocker, phentolamine also prevented the development of lymphopenia (Table 6.4; Fig. 6.3). In fact, adrenaline resulted in significantly raised levels of circulating lymphocytes (Table 6.4). Phentolamine cause a gross increase in spleen size and weight, due to engorgement with blood, in two experiments.

Table 6.1.

Effect of 3h intense acute stress or 3h stress followed by 1 week recovery on lymphocytes of farm-reared fish.

TREATMENTS	LYMPHOCYTES (k/μl)	STRESSED AS % CONTROL	CORTISOL (ng/ml)
A. CONTROL	25.9 ±1.5	100%	<1.0
STRESS (3hr)	12.9 ±1.0**	50%**	35±13**
B. CONTROL	30.5±2.7	100%	<1.0
STRESS (3hr)	20.0±2.0*	66%*	34±13**
RECOVERY (6d)	23.8 ± 3.5**	78%	<1.0
C. CONTROL	27.9±0.4	100%	<1.0
STRESS (3hr)	19.2±1.3**	69%*	15±1.24*
CONTROL	26.0 ± 1.3	100%	2.7±1.24
RECOVERY (6d)	25.0 ± 1.8	92%	<1.0
D. CONTROL	29.8 ± 1.5	100%	8±3.3
STRESS (3hr)	20.0 ±1.6*	67% *	27± 6.1**
RECOVERY (6d)	35.5 ±3.3	119%	9±3.3

Table 6.1A-D. Fish were stressed by 3h confinement, with noise during the first hour They were killed at the end of the stress period, or left 6 days to recover. (n=5)

* P<0.05 ** P<0.01 compared to control.

Table 6. 2.

Effect of repeated, daily stress on lymphocytes numbers of farm-reared fish.

TREATMENTS	LYMPHOCYTES (k/μl)	STRESSED AS % CONTROL	CORTISOL (ng/ml)
1. CONTROL	28.8 ± 1.6	100%	3.0 ± 0.9
CHRONIC (noise+confinement)	21.4 ± 1.0**	74%**	7.5 ±2.0
2. CONTROL	29.8 ±1.5	100%	8 ±3.3
CHRONIC (2x daily low water)	20.0 ± 1.8*	67%*	2.6 ±0.78

Table 6.2. Fish were either stressed by 3h noise plus confinement (Expt. 1) or by low water stress twice daily (Expt. 2) for 6 days in both cases and lymphocytes were counted after 3h from the last stress. (n=5; Student's 't' test).

* $P<0.05$ ** $P<0.01$ compared to control

FIGURE 6.1

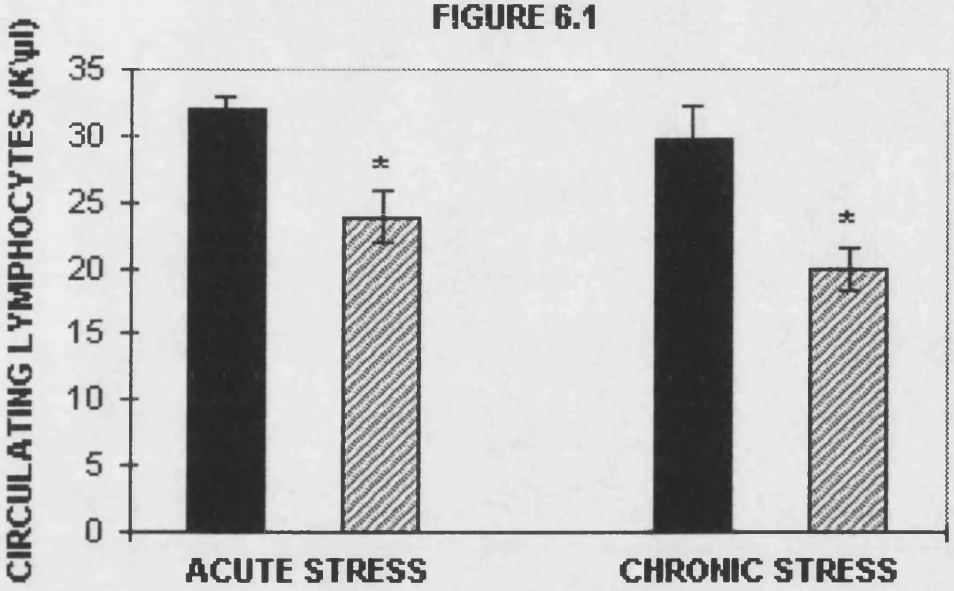


Figure 6.1. Fifty microlitre blood was mixed with 1:3 diluted Rees-Ecker solution. The number of lymphocytes was counted in a haemocytometer and expressed as thousands of cells/µl. Control values are shown by solid bars and stressed values by hatched bars.

Acuted stress was an injection given to mildly anaesthetised fish 3h before killing the fish (Table 1A-D).

Chronic stress was daily noise/confinement over 6 days period (Table 2).

n=5; * $P<0.05$ compared to control values.

Table 6.3.

Effect of injecting saline or cortisol on plasma lymphocytes, measured 3hr after the injection.

TREATMENTS	LYMPHOCYTES (k/μl)	STRESSED % CONTROL	CORTISOL (ng/ml)
A. CONTROL	30.0 ±1.0	100%	6.3 ±0.8
ANAESTHESIA	21.1 ±2.1*	70%	17.5 ±8.7
ANA+SALINE	20.5 ±1.4*	68%	16.9 ±12**
ANA+CORTISOL	31.8 ±1.3	106%	74.0 ± 12**$#
B. CONTROL	32.0 ± 1.0	100%	6.3 ±1.0
SALINE (without ana.)	23.9 ± 2.0**	75%	20.0 ±2.8*
CORTISOL (without ana.)	34.9 ±1.4	109%	59.8 ± 2.7**#

Table 6.3. Fish were anaesthetised (6:10,000) or not and injected with saline, or cortisol (50μg/0.5 ml saline).

Ana.= anaesthesia

* P<0.05 ** P<0.01 compared to unanaesthetised control.
# P<0.01 compared to saline injection.
$ P<0.05 compared to anaesthetised control.

FIGURE 6.2

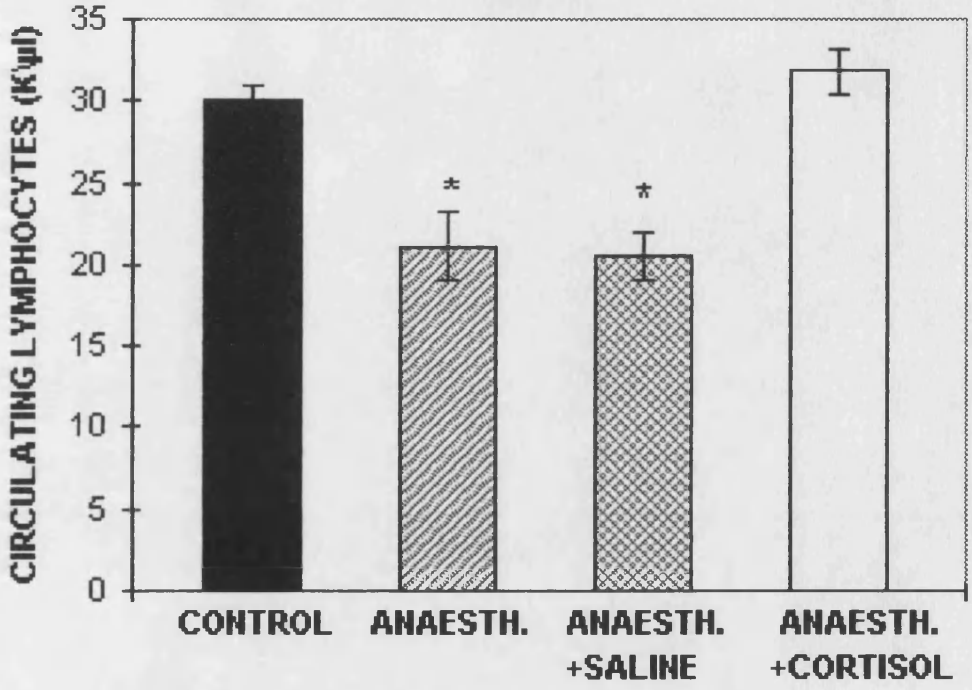


Figure 6.2. Fish were anaesthetised in phenoxyethanol (6:10000 v/v) and either then returned without further treatment or injected with 0.5ml saline (0.8% NaCl) or cortisol (50µg/0.5ml). They were killed 3h after recovery. Control values shown by solid bars; anaesthetised values by single hatched; anaesthetised plus injected by crossed-hatched and anaesthetised plus cortisol by empty bars.

n=5 * $P<0.05$ compared to control

Table 6.4.

Effect of injecting an adrenergic agonist or antagonist on lymphocytes determined 3hr after injection.

TREATMENTS	LYMPHOCYTES (K/μl)	STRESSED AS % CONTROL	CORTISOL (ng/ml)
CONTROL	24.5 ± 1.0	100%	8.3 ± 3.3
SALINE	19.3 ± 0.8**	79%	27.5 ±6.7*
NOR-ADRENALINE	31.4 ± 2.0#*	128%	6.2 ± 1.5
PHENTOLAMINE	22.3 ± 1.4	91%	42.7 ± 6.8

Table 6.4. Some farm fish adapted on white tanks were anaesthetised and injected with saline (0.8%), nor-adrenaline (2.5mg/0.5 ml saline) or phentolamine (5 mg/0.5 ml saline) (n=5; Student 't' test).

* P<0.05 ** P<0.01 compared to control.
# P<0.01 compared to saline.

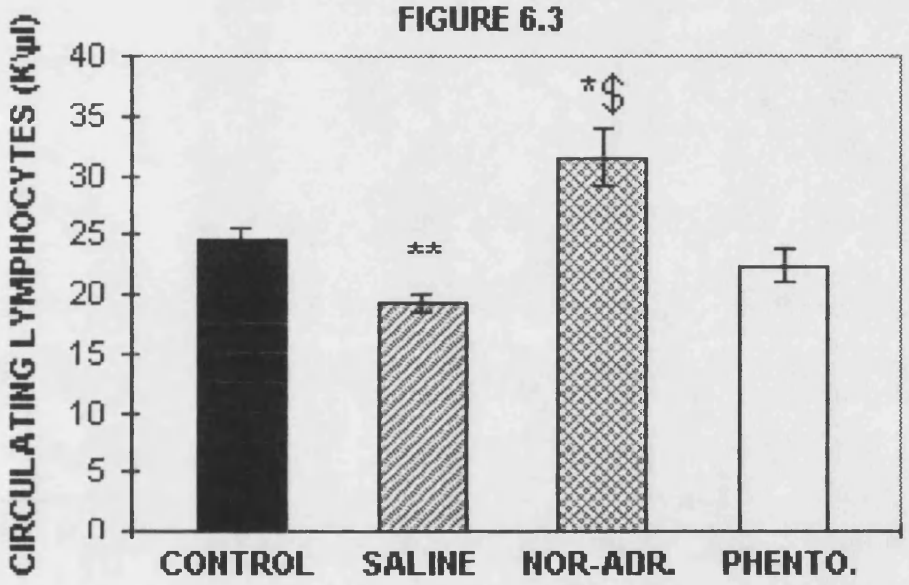


Figure 6.3. Fish were not anaesthetised. They were injected with either saline (0.8% NaCl), nor-adrenaline (2.5mg/fish) and phentolamine (5mg/ml). All in 500µl saline. Control values are shown by solid bars; saline injected by hatched; nor-adrenaline by crossed-hatched and phentolamine by empty bars.

n=5; * $P<0.05$ ** $P<0.01$ compared to uninjected control.
$ $P<0.05$ compared to saline.

6. 3. 1. Macrophage response.

The change in macrophage response was monitored in only one stress condition, i.e. heat stress. In contrast to the lymphocytes, exposure of fish to a heat stress at 25 °C resulted in a significant increase in the number of presumptive phagocytic cells in the blood (identified by their large and darkly staining nucleus) within 3 hr of stress ($P<0.01$; Table 6.5).

6. 3. 2. Changes in naturally infected fish.

In fish provided by the farm as bearing some infection, because of dark and solitary behaviour. Plasma cell counts showed an increase in circulating lymphocytes compared with the control (Table 6.6). Plasma cortisol and α-MSH values were not changed, but spleen weight was significantly increased, whether expressed as mg/fish (Control: 0.24±0.01; Infected 0.45±0.06; n=6) or as g/gbw (Control: 1.3±0.2; Infected 2.7±0.3).

6. 3. 3. Lymphocytes and background colour

Fish were reared in the aquarium, rather than being imported from a fish farm as in the cases discussed above. Basal lymphocytes numbers in fish reared in white or black tanks were similar, and not apparently influenced by tank colour, nor different from basal levels in farm-reared fish.

When stressed by a method equivalent to that used on farm fish (injection stress or 3h noise plus confinement), they responded in a similar way i.e. by lymphopenia in both

Table 6.5.

Effect of temperature stress on leukocytes.

TREATMENT	LYMPHO. (K/μl)	STRESSED % CONTROL	MACRO. (K/μl)	STRESSED % CONTROL	CORT. (ng/ml)
CONTROL	30.4±0.6	100%	3±0.6	100%	0.6±0.1
TEMP. STRESS (25°C)	20.0±0.6**	66%	7.6±0.7*	177%	21±2.4**

Table 6.5. Fish were stressed by transferring into a warm water at 25°C till unconscious for approximately 5 minutes and then returned to their home tank. They were killed 3h after stress. Lymphocytes and macrophages were measured in the blood (n=5; Student's 't' test)

* $P<0.01$ ** $P<0.001$ compared to control.

Table 6.6.

Effect of infection on immune parameters.

GROUP	LYMPHOCYTES (K/μl)	SPL. WT (mg/gbw)	CORTISOL (ng/ml)	α-MSH (pg/ml)
CONTROL	19.4±0.9	1.3±0.2	2.3±1.3	560±146
INFECTED	31.2±2.9*	2.7±0.3**	3.3±1.2	588±73

Table 6.6. Presumed infected trout and control trout were brought from the fish farm and kept in the aquarium for 7 days. n=6.

* $P<0.02$ ** $P<0.01$ compared to control.

Table 6.7.

Response of lymphocytes from fish reared on white or black backgrounds to various stresses.

		WHITE-REARED			BLACK-REARED	
TREATMENTS		**LYMPHO. (K/μl)**	**CORTISOL (ng/ml)**		**LYMPHO (K/μl)**	**CORTISOL (ng/ml)**
A. INJECTION + 3H RECOVERY	**WC**	30±2.1 (100%)	2±0.5	**BC**	27±2.6 (100%)	>1.0
	WS	19±2.2** (63%)	15±6.3*	**BS**	17±1.2** (63%)	21±5.8**
B. 3H NOISE + CONFINEMENT	**WC**	25±1.6 (100%)	1.2±0.4	**BC**	21±0.7 (100%)	1±0.2
	WS	18±1.6* (72%)	16±3.9**	**BS**	16±0.6** (76%)	34±5.4**
C. 2 MIN AIR STRESS+2H RECOVERY	**WC**	22±1.7 (100%)	13±1.8	**BC**	25±2.9 (100%)	>40
	WS	37±4.8* (168%)	73±10.0**	**BS**	22±0.9 (88%)	98±7.4**
D.	**WC**	25±0.9 (100%)	1±0.12	**BC**	24±1.0 (100%)	1±0.2
3 MIN LOW H_20 +4H RECOVERY	**WS**	28±0.6* (112%)	32±8.1**	**BS**	25±0.7 (104%)	34± 8.4**
28H RECOVERY	**WS**	18±1.0** (72%)	5±0.7*#	**BS**	21±0.8 (88%)	12±2.8*#

Table 6.7 Aquarium-reared fish on white and black backgrounds were stressed by saline injection (Table 7A) or 3h noise plus confinement (Table 7B) and killed 3h after stress initiation . Some fish were hold in a net for 2 min and killed after 2hr (Table 7C); others were stressed in low water for 3 min and then killed at 4hr and 28 hr after the stress (Table 7D). Numbers in brackets are stressed as % of control. (n=5).

* P<0.05 ** P<0.01 compared to control
# P<0.05 compared to 4h stress.

WC = White-Control
WS = White-Stress
BC = Black-Control
BS = Black-Stress

white-reared and black-reared fish (Table 6.7 A, B). However, when they were stressed of water for 2 min or by being placed in very shallow water for 3 minutes, white-reared fish showed an increase in circulating lymphocytes (Table 6.7 C & D), while black-reared fish failed to respond at all.

6.4. DISCUSSION

Previous work on mammals has shown that the number of circulating lymphocytes may either increase (Edwards et al., 1984; Maisel et al., 1990) or decrease (MorrowTesch et al., 1993; Dhabhar et al., 1994) in response to stress. The present study demonstrates that in fish, also they may increase or decrease in response to stresses. An increase in lymphocytes was observed on only two occasions and a decrease on all others. This increase was found when fish were stressed either by holding them in air or by exposing them to shallow water. This procedure may have been stress by cutting the oxygen supply, whereas their decline was noticed when fish were stressed either by injection, anaesthesia, 3h noise plus confinement or warm water. The reason why some stresses should cause an increase and other decline is not clear. Judging from the increase in cortisol levels it was apparently not due to the severity of stress. However, Pickford et al., (1971) investigated the effect of epinephrine and cortisol on leukocytes numbers in *Fundulus heteroclitus* and reach the conclusion that an initial rise (15 min) is casued by β-adrenergic stimulation and the later rise in leukocytes (mainly lymphocytosis) seen 2 hr later was a response to cortisol (0.025 μg/gbw), since it was blocked by hypophysectomy or administration of metapirone (cortisol synthesis blocker) and induced by high (but not low) doses of ACTH in hypophysectomized fish. In trout, cortisol (0.25μg/gbw) restored circulatory lymphocytes to control level, thus preventing the decline which results from injection stress, but did not cause lymphocytosis.

Murray et al., (1992) and Maisel et al., (1990) have pointed out that exercise stress, which increases lymphocytes in the circulation, was associated with increase in

catecholamines and this increase in lymphocytes was mediated through β-adrenerigc receptors. These authors have also found that this effect evident after 15-20 min of exercise stress, was blunted by prior administration of propranolol, suggesting that this early increase was mediated through β-adrenergic receptors. There was no increase or decrease of lymphocytes in response to α-adrenergic agents (Murray et al., 1992). This suggested that changes in lymphocyte migration were mainly mediated through β-adrenergic system. The presence of β-adrenergic receptors on lymphoid cell has been demonstrated in mammals (Brodde et al., 1981) and their number and density varies according to the cell subtypes (Maisel et al., 1990). Moreover, activation of sympathetic nervous system via exercise stress (Maisel et al., 1990; Murray et al., 1992) as well as exogenous administration of epinephrine (Crary et al., 1983), or isoproterenol (van Tits et al., 1990) have been shown to increase β- adrenergic receptors, causing lymphocytosis (Maisel et al., 1990; Murray et al., 1992).

In the present study, administration of very large doses of nor-adrenaline to trout increased lymphocytes 3h later. Whether this lymphocytosis, seen here at a later period than in mammals and *Fundulus heteroclitus* is due to adrenaline itself or to other agents triggered by the high catecholamine dose, is not clear. No receptor blocker was administered with the adrenaline to determine which receptor type was involved. But so far as lymphocytopenia or lymphopenia in fish is concerned, other author have shown the involvement of both α- and β- adrenergic receptors. Thus, isoproterenol caused decrease in their numbers and administration of propranolol altered the early (15 min) leukocytosis (Pickford et al., 1971b). In the same study, these authors have shown that lymphopenia caused by epinephrine at 3 min and 45-60 min was abolished by α-adreneregic

antagonist, phentolamine and phenoxybenzamine. But again, the alteration of these phases of lymphocytes were found in early phases of adrenergic agent administration, suggesting involvement of adrenergic receptors in early regulation of lymphocytes.

To explore the potential cause of lymphocytosis or migration of lymphocytes into the circulation, results may be interpreted in the following way: Lymphocytes have two types of homing receptors, one is 'E-selectin' which causes weak adhesion of lymphocytes to the endothelial cells lining post-capillary venules in the lymph nodes, and activation of the other receptor type, 'Integrin' causes their strong adhesion (Alberts et al., 1994). This adhesion of lymphocytes can be dissociated under physiological conditions (Stamper and Woodruff 1977). Therefore, it is possible that stress-induced activation of β- adrenergic system may have disrupted these homing receptors, resulting in lymphocyte release into the circulation. Alternatively, the existence of adrenergic innervation of mammalian (Felten et al., 1987) and fish spleen (Nilsson and Grove 1974) is evident and this local innervation may be the source of release of catecholamines. The action of α-, β- and cholinergic agents have all been shown to regulate the splenic contraction in fish (Holmgren and Nilsson 1975). Again, contraction of spleen in fresh water teleost under exercise stress (Yamamoto, 1988) may be caused by circulating catecholamines released in the blood from the kidney chromaffin cells (Randall and Perry 1992). Therefore, it is possible that changes of lymphocytes may be attributable to one or other reasons stated above.

The observed decrease in lymphocytes however, in other cases of acute stress may be due to their migration to other lymphoid organs such as spleen, pronephros, thymus or

non-lymphoid organs such as intestine or general body stroma. Alternatively, they could have remained in the circulation but, due to catecholamines or other hormonally mediated stress-induced osmoregulatory dysfunction, plasma volume may have increased so that their concentration per microlitre simply decreased due to the blood dilution. However, in view of the increase in presumptive neutrophils during stress, this potential explaination seems unlikely. It is also possible that immediate release of catecholamines may have increased their numbers and the cortisol may have its effect in the longer term, as Pickering (1984) in trout have shown the decrease in lymphocytes after 48 hrs of exogenous cortisol administration in trout.

A marked increase in splenic mass by phentolamine treatment indicates the role of catecholamines in the regulation of blood flow. If stress-induced catecholamines contracts the spleen, releasing lymphocytes in blood then, phentolamine may permit their migration back to spleen. This was confirmed in a most recent mammalian study by Madden et al., (1994). These authors demonstrated the migration of radiolabelled lymphocytes from circulation into the lymph nodes in chemically sympathectomized mice. This suggested that elevation of endogenous catecholamines may causes their migration into the circulation.

The number of circulating lymphocytes seen in response to repeated stress was same as that seen in response to acute stress. Repeated stress for 6 days may have down regulated their β- receptors, inhibiting their release into the blood. In mice chronic α-adrenergic stimulation by endogenous catecholamines has been shown to cause lymphopenia (Maisel et al., 1990). Whether this lymphopenia is caused by direct

antiproliferative effects of the α_2- adrenergic stimulation, reducing their numbers in the body or by restricted immunoregulatory cell traffic between lymphoid organs and the circulation was not known. Thus, an increase or decrease in lymphocytes in response to different stresses may be related to density of α- or β- adrenergic receptors, dictating which of these two systems is activated.

Number of phagocytes identified on the basis of nuclear size and staining properties, was found to increase in response to warm water stress. This increase may from the release of monocytes and neutrophils from storage in the spleen and kidney. Recent study by Pulsford et al., (1994) have shown that an increased phagocytes number in response to shallow water and handling stress. However, their increase or decrease into the circulation may depend upon the amount of catecholamines released and their sensitivity to catecholamines as Crary et al., (1983) have shown that different cell types have different sensitivity to epinephrine.

6. 4. 1. Cortisol and its effect of lymphocytes .

To explore the potential cause of lymphocytosis or lymphopenia, fish were administered with cortisol. Administration of cortisol normally depresses plasma level of lymphocytes examined 2-3 days later (Pickering 1984; Pickering et al., 1987). Surprisingly, administration of cortisol prevented the decline in lymphocytes caused by injection stress. It is possible that the effect of cortisol on lymphocytes, seen in the present study, is an example of feedback mechanism. Increased plasma cortisol may

have feedback onto the HPI axis to reduce the response of ACTH and cortisol on lymphocytes.

6. 4. 2. Effect of infection on immune parameters.

One of the responses to infection seen in the present study was an increase in lymphocyte numbers in the blood in parallel with an increase in spleen size and weight. These increased parameters may be due to the lymphocyte proliferation induced by infectious organisms. Thus, foreign antigens selectively stimulate lymphocyte proliferation and maturation (Alberts et al., 1994). In such fish, however, plasma levels of cortisol and α-MSH were not affected.

6. 4. 3. Lymphocytes and background colouration.

Lymphocyte counts in white-reared fish showed either an increase or decrease in numbers in response to stress. Lymphocytes were increased in response to 2 min air or 3 min shallow water stress. Whereas, their numbers were decreased in response to injection, anaesthesia or 3h noise stress. In black-reared fish, however, a rise in response to stress was not observed.

On white background, stress induced release of MCH is more than in black-adapted fish (Green and Baker 1991). Thus, under stressful conditions MCH will depress CRF (Green et al., 1991). Whether increased MCH levels in fish on white background

modulated the autonomic nervous system in such a way that the balance of α- and β-receptors was different from that seen in black-reared fish, is not known.

CHAPTER 7

GENERAL DISCUSSION

The work described in the preceeding chapters has led to a number of observations which may have wider implicatoins in aquaculture practices. On other hand, in some it cases poses further problems and require more research. Firstly, the technique (phagocytosis of yeast cells, which has been expresed in terms of PI) which has been used in the present study is simple, easy and less laborious than some alternative methods and involves the use of only a few slides, stains and yeast cells and give a significant result when measured under mild stress. Additionally, this technique can be used in field studies to monitor the effect of environmental stresses such as rise in temperature and pollution etc.

It was observed that acute stress, lasting 2 min to 3h, caused a rapid decline in the number of phagocytic events by macrophages extracted from the spleen and the pronephros. Under stressful conditions there was significant increase in the number of macrophages which did not engulfed any yeast cells. This suggests that stress might depress the number of pathogens such as bacteria that could engulfed by macrophages in the two lymphatic tissues studied here- spleen and pronephros. Since, exposing fish to either 2 min handling stress or 3h transportation plus confinement are normal and routine practices in aquaculture. The immune system of farmed fish may be compromised. This might result in detrimental consequences on fish health. Results from this study may have practical implication for aquaculture.

However, from above study, the question arises what causes the apparent decline in phagocytic activity in both organs? It is not clear whether stress, applied in the present study, affects the activity of macrophages only at the individual cell level, or if it changes the composition of cells which contribute to the overall macrophages population in the two sites.

To explain the cause of decline in phagocytic activity, a number of possible interpretations can be put forward: Firstly, the depression in phagocytic activity of macrophages may be due to the emigration of active macrophages out of the pronephros and spleen to other organs, such as intestine, thymus, blood or general body stroma, leaving only inert or less active macrophages. If macrophages were to leave these organs, then one would expect their apparent increase in the blood or other tissue. Possibly, after 3h of acute stress, using the differential blood count together with a reliable macrophage marker, the number of macrophages might be shown to increase in the circulation. They may contribute to the new population of cells observed in the blood stream after stress, whose precise nature could not be determined in the present work. Secondly, radioactive material or colloidal carbon introduced into the blood stream would be phagocytosed by pronephric and splenic cells; measuring the radioactivity in control fish or 3h after acute stress, might reveal if relocation or re-distribution phenomenon occurred in fish under stress.

In mammals, loss of white blood cell adhesiveness can occur through the adhesive molecules such as 'selectin' and 'integrin' (Alberts et al., 1994). It is also possible that fish phagocytes may have lost their adhesiveness, so that they no longer became attached to

the glass slides. Although, they continue to reside in the spleen/pronephros This could be explored by flooding the slides with a splenic and pronephric cell suspension and challenging them with magnetic beads which will be engulfed by macrophages. By passing a magnet beneath the slides, non-adherent macrophages with engulfed magnetic beads can be collected and measured.

Another anomaly observed in the present study was that the administration of cortisol, surprisingly, prevented both the decline of plasma lymphocytes, and the decline in phagocytic activity which occur in response to stress. Changes in adhesiveness of these cells after stress in the presence or absence of cortisol administration is thus of potential interest. We have already proposed that adrenaline/nor-adrenaline is a mediator of stress. It is possible that the cortisol injection had a fast-feed back effect on the hypothalamus and perhaps depresses the expected increase in plasma adrenaline or the release of sympathetic adrenaline within the spleen tissues. To investigate this, it would be necessary to measure plasma and tissue catecholamine levels. This was not attempted in the present study because of the cost of such measurements, and the large amount of plasma (at least 2ml) which is needed.

In the present study, when stress was administered once and the phagocytic activity was assessed after 24hr, one week, and two weeks, the phagocytic activity was found to be depressed after 24h, and surprisingly, increased after a week and then resembled the controls in the second week. This result may have practical implications in fish culture

system which implies that following a week of acute stress, fish may cope strongly with pathogens by generating 'new' and 'active' macrophage population. But again, the question that arises in this case is what causes the enhancement of macrophage phagocyte activity? Theoretically it could be that stress may have stimulated the haemopoietic tissue to generate a 'new' population of macrophages. It might be possible to examine this by injecting fish with a mitotic marker such as ^{3}H thymidine, to determine whether stress results in an increased number of labelled phagocytes.

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SECTION II

IMMUNOCYTOCHEMISTRY

CHAPTER 8

THE SECOND GROUP OF MELANIN-CONCENTRATING-HORMONE (ir-MCH) IN TROUT BRAIN AND ITS RESPONSE TO BACKGROUND COLOURATION.

8.1. INTRODUCTION

Melanin-Concentrating-Hormone (MCH), is a neuropeptide that has been demonstrated in the brain of many vertebrate classes. This includes lampreys (Baker, 1991), elasmobranchs (Vallarino et al., 1989), teleosts (Rance and Baker 1979; Kawauchi et al., 1983; Bird et al., 1989; Groneveld et al., 1995), amphibia (Anderson et al., 1986), reptiles and Birds (Cardot et al. 1994) and mammals, including rat (Bittercourt et al., 1992) and the human (Bresson et al., 1989).

In fish, this neuropeptide was first investigated because of its ability to change the skin colour according to the background (Rance and Baker 1979; Baker and Rance 1983; Kawauchi et al., 1983; Baker, 1988). MCH is produced in magnocellular neurones located on the floor of the hypothalamus near the pituitary stalk, in the lateral region of the nucleus lateralis tuberis (NLT). Using a hybridization study, the expression of MCH-mRNA expression has also been demonstrated in hypothalamic neurones (Baker et al., 1995; Groneveld et al., 1995). Although the cell bodies are restricted to the hypothalamus, their axons are widely distributed throughout the brain including the preoptic region, pretectal region, either side of the posterior commissure and also in the

spinal chord (Naito et al., 1985; Bird et al., 1989). The wide distribution of fibres in almost all parts of the brain suggest that this neuropeptide may have neurotransmitter/neuromodulatory role in brain.

In most species of vertebrates, very few axons project to the posterior pituitary lobe, but in teleosts, the pituitary neurohypophyseal lobe is the main projection target for MCH fibres (Naito et al., 1985; Bird et al., 1989) where this neuropeptide is stored (Barber et al., 1987).

In fish, MCH plays an important role in regulating the colour of the skin by its ability to cause pigment aggregation within the melanophores, xanthophores, and the erythrophores (Fuji and Oshima 1986; Castrucci et al., 1988; Baker, 1991). The abundance of immunoreactive MCH, its synthesis in the brain and release from the brain and the pituitary gland of trout depend on the colour of the tank in which the fish is kept (Kishida et al., 1989; Baker, 1991). These changes in the pituitary content of MCH in response to background colour have been also observed in other species of fish such as eel, *Anguilla anguilla* (Powell and Baker 1988) and the grass carp, *Ctenopharyngodon idellus* (Bird and Baker 1989) suggesting it is used in colour regulation in all these species.

MCH antagonizes the action of the other colour regulating hormone, α-melanocyte-stimulating-hormone (α-MSH), at the level of the melanophore (Baker, 1988; Svensson et al., 1991) and also reduces its release from the pituitary intermediate lobe (Baker et al., 1986; Barber et al., 1987). In additon, it depresses the (CRF)-induced release of

ACTH from the pars distalis acting in a paracrine manner (Baker et al.,1985) and also appears to depress the release of CRF from the hypothalamus (Green et al., 1991). Thus in teleosts, this neuropeptide acts in a several ways, not only regulating skin colour but also influencing the response to stress to contribute to the regulation of skin colour.

Physiological pigmentary changes in teleost fish can be influenced by several agents, including not only the two colour-change hormones (MCH which causes pallor, and its antagonist α-MSH which induces darkening), but also neurotransmitters from the autonomic nervous system, i.e nor-adrenaline (Green and Baker 1989).

Not only does MCH apparently influence the hypothalamo-pituitary-adrenal (HPA) axis, but stress and glucocorticoids can also affect the release and synthesis of MCH (Green and Baker 1991; Baker and Bird 1992). Daily stresses are stimulatory to MCH release and synthesis (Green and Baker 1991; Baker and Bird 1992), but more frequent and prolonged stresses appear to depress MCH synthesis (Baker and Bird 1992).

In mammals, the wide distribution of MCH fibres throughout the brain, and the location of MCH perikarya in several parts of the hypothalamus (Bittencourt et al., 1992) suggest that MCH and the related peptide such as Neuropeptide Glutamine (E) Isoleucin (I) (NEI) derived from the same precursor influence a wide range of brain activities. Most MCH perikarya are located in the lateral hypothalamic area (LHA), which lesioning or electrical stimulatory experiments suggest may serve as a sensorimotor integrative centre, associated with the modulation of behaviours such as eating, drinking, with arousal and motor activity (Bittencourt et al., 1992). Other MCH neurones occur in the

anterior hypothalamic region and are seen exclusively during lactation (Knollema et al., 1992). Their precise role in lactation and maternal behaviour is not yet known.

In rat auditory stimulation by sound will evoke an electrical potential in the pyramidal layer of the dorsal hippocampus which is enhanced by the neuropeptide α-MSH, hippocampal activity is influenced by input from the medial septal nucleus which is rich in MCH and α-MSH neurones. An application of MCH will antagonized the firing rate evoked by auditory stimuli, and oppose the effects of perfusion of α-MSH (Miller et al., 1993) suggesting that MCH may have an influence on behavioural changes occuring in response to auditory stimuli.

The role of MCH in drinking and fluid homeostasis came from the observation that either dehydration or allowing rats to drink 2% saline will increase the MCH concentration in the lateral hypothalamus and pituitary, suggesting a reduction in MCH release. There is an associated overall reduction of MCH-mRNA (Nahon et al., 1993; Presse and Nahon 1993; Fellman et al., 1993). In addition, MCH gene expression is also influenced by stress and glucocorticoids (Presse et al., 1992).

The use of modern techniques such as polymerase chain reaction, Northern blotting and *in situ* hybridization all show that cells expressing MCH-mRNA can be detected in peripheral lymphoid (spleen) and non-lymphoid tissues such as the intestine, thyroid, and testis (Breton et al., 1993; Nahon et al., 1993). This suggests that MCH may also have roles outside the nervous system.

The work in the present chapter was initially undertaken to learn some immunohistological techniques, such as immunocytochemistry (ICC) and sectioning techniques, such as use of the vibratome. The study revealed the presence of a hitherto unknown group of MCH neurones, located above the lateral ventricular recess. This histological work has also studied whether these parvocellular neurones will respond to background colour, in the same way that has been shown for the magnocellular neurones of the NLT.

8.2. MATERIALS AND METHODS

8.2.1. Fish.

Immature rainbow trout (*Oncorhynchus mykiss*) weighing 40-60 g were obtained from a local fish farm (Alderely Trout Ltd Wotton Under Edge, Glouc.), and kept in white tanks in flowing water at 10-12 °C. All fish were kept under a photoperiod of 16h:8h light:dark.

In addition rainbow trout eggs were obtained from Bibury Trout Hatchery, Glouc., and reared in the university aquarium in running well water and used at about 40-60g or about 200-300g (18 months old).

8.3. Tissue Preparation and Immunocytochemistry.

8.3.1 Vibratome Sections.

Fish were deeply anaesthetised in phenoxyethanol (6:1000 v\v) and perfused through the heart with phosphate buffered saline (PBS, 0.01M, pH 7.8) containing heparin, followed by Stefanini's picric acid, paraformaldehyde solution (Stefanini, 1967) at 20 °C over 10 min (40 ml). The perfused brains were left in the same fixative overnight at 4 °C. Fixed brains were embedded in glycine gelatine (15 ml glycerol, 16g gelatin, 70 ml H_2O) and 100 μm thick sections were cut on a vibratome. Sections were immunostained using the peroxidase-antiperoxidase (PAP) method. After washing in 10 mM PBS, pH 7.5, and pre-incubation for 30 min in 3% goat serum in PBS, free floating sections were

immunostained for 3 days at 4 °C with antiserum against salmonid MCH (Kawauchi) diluted x1000 in PBS containing 0.1% Triton X-100 and 3% normal goat serum (PBST). After thorough washing, sections were incubated for 2hr at room temperature with goat anti-rabbit globulin (Sigma Chemical Co., Poole, UK), diluted x25 in PBST, washed and incubated for 2h at room temperature in rabbit peroxidase-antiperoxidase (PAP) serum (Sigma), diluted x200 in PBST. After further washing in 10mM Tris-HCl solution, pH 7.5 (Tris buffer), sections were incubated first for 15 min in diaminobenzidine (DAB) solution (Sigma 25 mg/100 ml Tris buffer), and then in fresh DAB solution to which had been added 100 µl full strength H_2O_2 per 100 ml buffer. Sections were left for the enzyme reaction to proceed for 10-15 min before washing in PBS. They were then mounted onto gelatine-coated slides. Dried sections were dehydrated in graded alcohol, cleared in xylene and mounted in DPX.

8.3.2. Wax sections.

Trout of 40-60g or 18 months, reared on white or black backgrounds or transferred from white to black (W-B) and vice-versa for 6 days (only 40-60g fish), were perfused through heart as before, but using Bouin's fixative. Wax-embedded brains were sectioned transversely at 8 µm. Immunostaining was carried out by the same procedure as described above. The primary antiserum for this work was anti-rat MCH (Lot no. 837) raised by Eberle.

The sections were counterstained with haematoxylin and the nuclei of the MCH perikarya were drawn at x1000 magnification, using a camera lucida. Nuclear area was determined from these drawings.

Figure 8.1

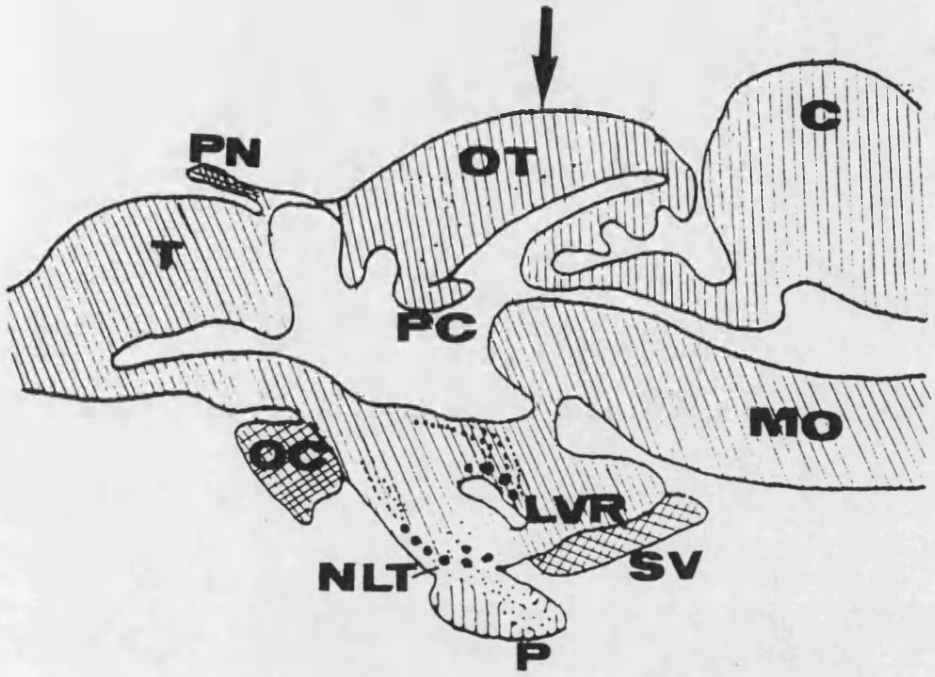


Figure 8.1. Outline drawing of a parasagittal section on the trout brain showing the location of the MCH cell bodies (large dots) and major fibres tracts (small dots). Arrow on the parasaggital section indicate the level of the transverse section of figure 8.2.

Abbreviations: T, Telencephalon; OT, Optic tectum; PC, Posterior commissure, C, Cerebellum, OC, Optic chiasma; NLT, Nucleus lateralis tuberis; P, pituitary; PN, Pineal gland; LVR, Lateral ventricular recesses; SV, Saccus vasculosus; VC, Valvula cerebellum; MO, Medulla oblongata.

Figure 8.2

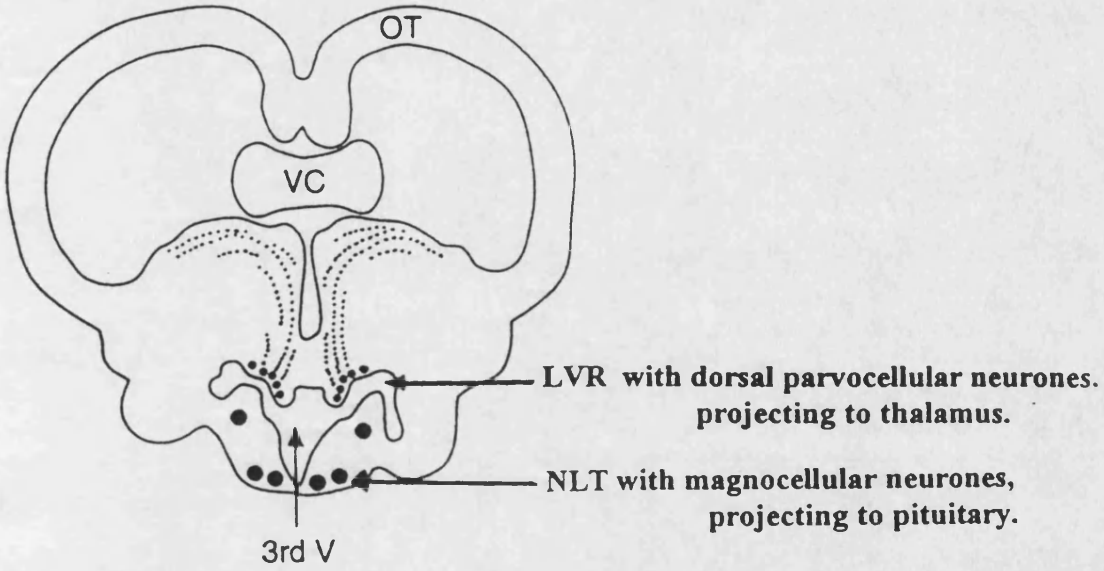


Figure 8.2 Diagram showing the location of parvocellular ir-MCH neurons above the lateral ventricular recess (LVR) and magnocellular ir-MCH neurons on the floor of the posterior hypothalamus, in the nucleus lateralis tuberis posterioris (NLT). The location of MCH neurons in the basal hypothalamus (large dots) and above the lateral ventricular recess (small dots) is shown. 3rd V= third ventricle; OT= Optic tectum; VC= Vulvula of cerebellum.

8.3. RESULTS

8.3.1. Immunocytochemistry of second group of r-MCH neurones.

Vibratome Sections:

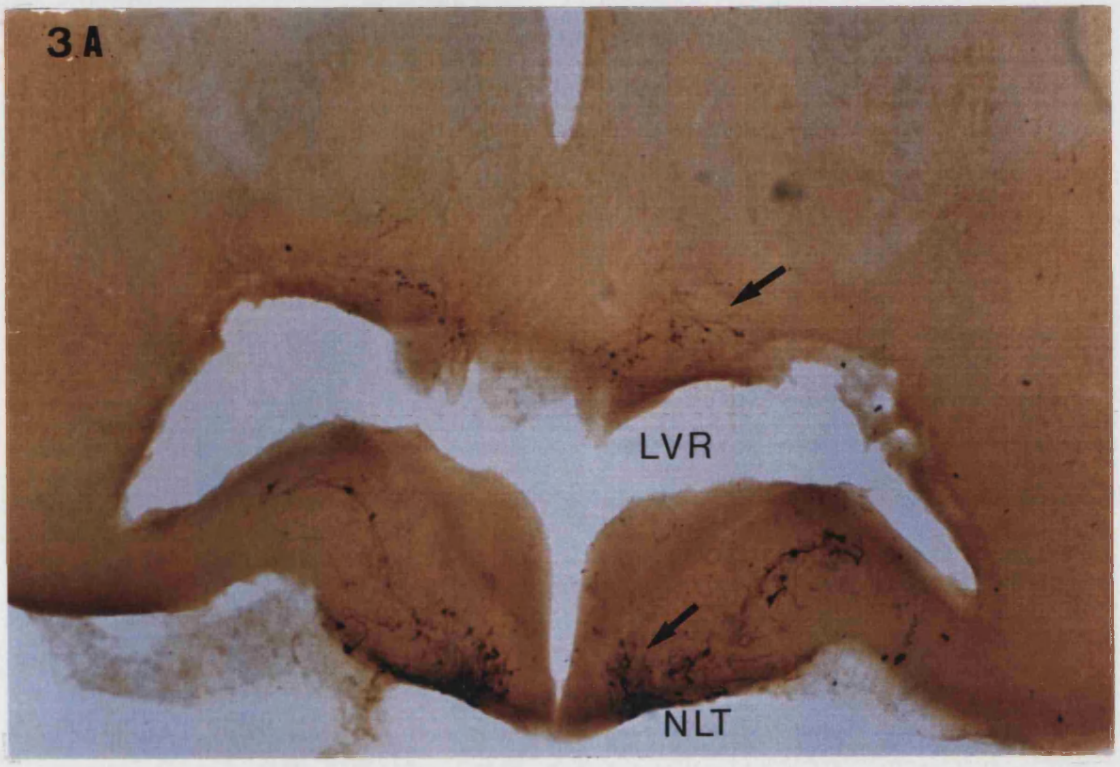
100 μm-thick immunostained vibratome sections through the trout brain show the location of two type of ir-MCH cell groups: One group of ir-MCH neurones is located on the floor of the hypothalamus in the NLTpl and NLTpp (See Diagram 8.1), that has been referred to as NLT-MCH neurones (Suzuki et al., 1995). Another, comparatively smaller group of parvocellular ir-MCH neurones is closely associated with paraventricualr organ (PVO), situated bilaterally above the lateral ventricular recesses (LVR) and has been called LVR-MCH neurones (Figs. 8.2, 3A & B).

This study concentrates on the second group of neurones, LVR-MCH. The neurones of this group appear to be bipolar, vary in size and are distributed over the dorsal part of the PVO (Fig 8.3A 4A). Smaller immunoreactive MCH cell, measuring on average 11.5 x 7.5 μm along their two axes, tend to be found more medially and closer to the 3rd ventricle of the LVR, while more dorsolaterally located MCH neurones tend to be larger (Fig. 8.3B) measuring about 18.5 x 12μm

Two major axonal tracts originate from these LVR-MCH neurones. One projects dorsally into the thalamus, coursing on either side of the third ventricle (Figs. 8.2; 3B) Other ir-MCH fibres are seen either side of the posterior commissure and either side of the the habenular. In addition, a second pair of short bilateral tracts appear to be directed

Figure 8.3A. Photomicrograph showing the location of parvocelluar and magnocellular ir-MCH cells (arrowed).

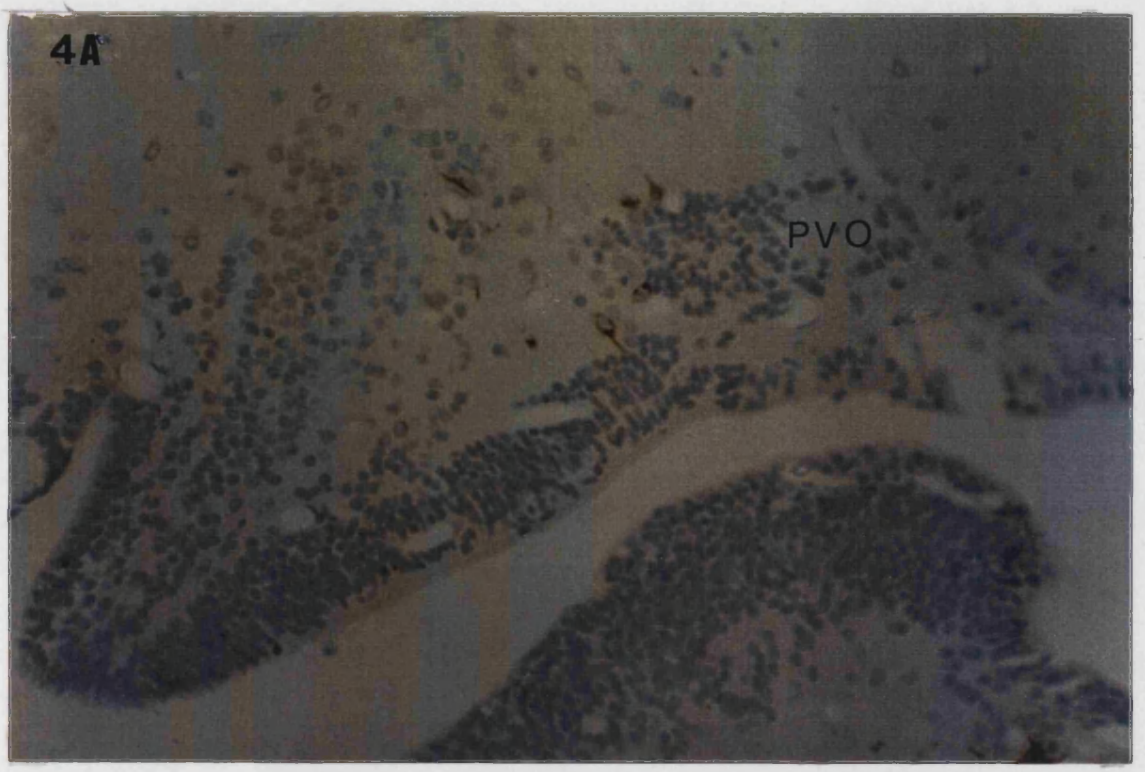
Figure 8.3B. The axons project dorsally to the thalamus. Magnification x1000.

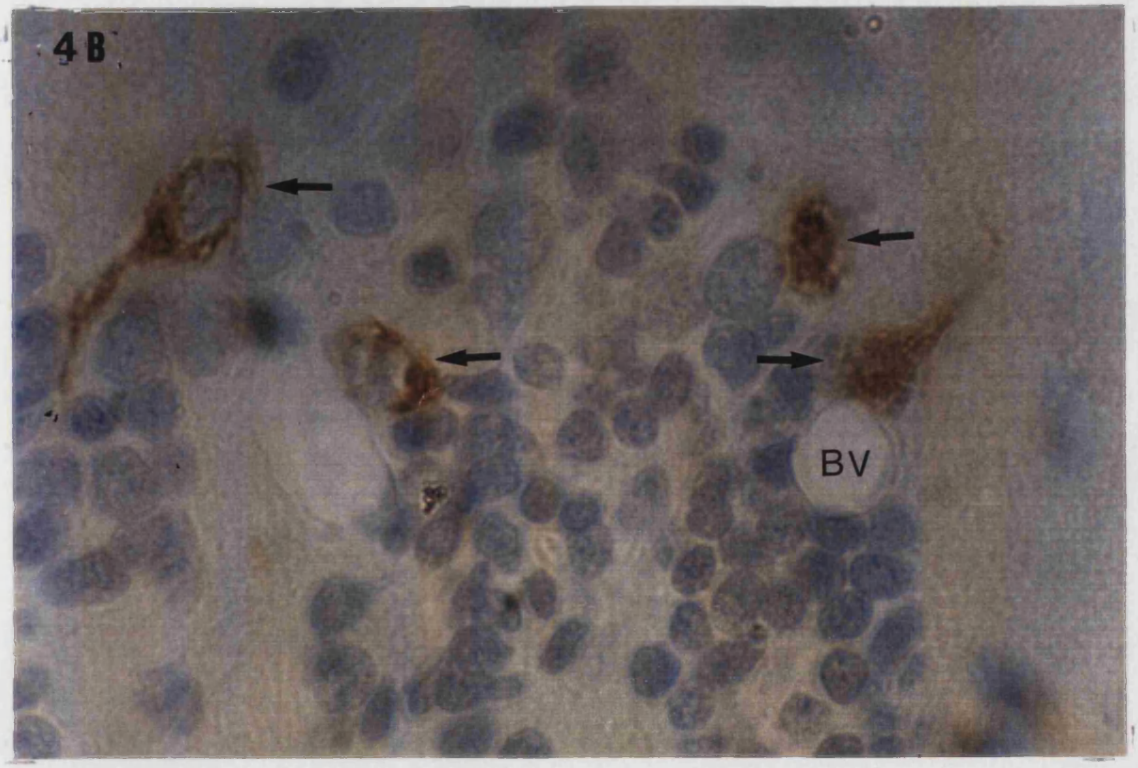

3A
LVR
NLT


3B

Figure 8.4A. Photomicrograph of paraventricular organ (PVO) above lateral ventricular recess (LVR) and the location of ir-MCH neurons in PVO. Some neurones are in close vicinity of blood vessels (BV) at x1000 magnification.

Figure 8.4B. Photomicrograph showing the enlarged view of the PVO with ir-MCH neurons, shown by arrows. x1000 magnification


4A
PVO


4B
BV

ventrally towards the ependyma of the ventricular cavity close to the posterior tubercle (Fig.8.3B). Whether they actually make contact with the ventricular cerebrospinal fluid or terminate on the well-developed capillary network in this region could not be determined from vibratome sections. However, the neurones of the LVR-MCH or fibres originating from them do not seem to contact the pituitary gland (Fig. 8.2).

8.3.2. Response of the second group of ir-MCH to background colouration.

Whether rearing conditions and age of fish have influence on LVR-MCH activity was assessed next. The fish were reared on white or black background (both young and old fish) or transferred them from black to white (B-W) or vice-versa for 6 days (only young fish). The activity of these neurones expressed in terms of either their number or nuclear size, was modulated by background colour. Results presented in table 8.1 suggests that ir-MCH cells were more numerous in fish on white background as compared to fish reared on a black background. However, transferring them from black to white (B-W) background for 6 days resulted in a significant increase in the number of visible ir-MCH cells which were now higher than in the control group (Table 8.1).

When their activity is expressed in terms of nuclear area, immunoreactive LVR-MCH neurones from trout reared in white tanks have larger nuclei than the LVR-MCH neurones from fish reared in B tank (Table 8.2, Fig 8.5 A, B). Fish transferred from white to black (W-B) tank for 6 days had apparently smaller LVR-MCH cell nuclei, although the reduction was not statistically significant. Fish transferred from black to white (B-W) for 6 days however, showed larger nuclear area (Table 8.2).

Results also showed that, compared to young fish, the nuclear size of neurones was significantly larger in older fish, reared on white or black backgrounds ($P<0.05$). The measured nuclear size of these neurones from 18 months white-reared (44.4 ± 2.4 μm2) and black-reared (24.5 ± 2.7 μm2) fish was greater than that seen in young fish (White-reared, 34 ± 3.5; Black-reared fish 14 ± 1.1).

The increased MCH content in white-reared trout, was suggested by the more abundant ir-MCH granulation, confirmed by radioimmunoassays of MCH in pituitary, NLT and LVR regions (Table 8.3).

Table 8.1.

Number of ir-MCH neurones in young trout reared on white or black background.

BACKGROUND	REGION OF HYPOTHALAMUS	
	LVR	NLT
WHITE-REARED	67 (42, 25)	362 (182, 180)
BLACK-REARED	45 (21, 24)	119 (63, 56)
B-W (6 days)	157 (86, 71)	612 (312, 300)

Table 8.1. The brain from a single fish from each background condition was sectioned on a vibratome, immunostained for MCH, and the neuronal cell bodies in the NLT and LVR were counted. Numbers of neurons on each side of the brain are shown in brackets.

Table 8.2

Change in the area of ir-MCH cell nuclei of fish reared on white or black or change of background Values are cell nuclear area (μm2).

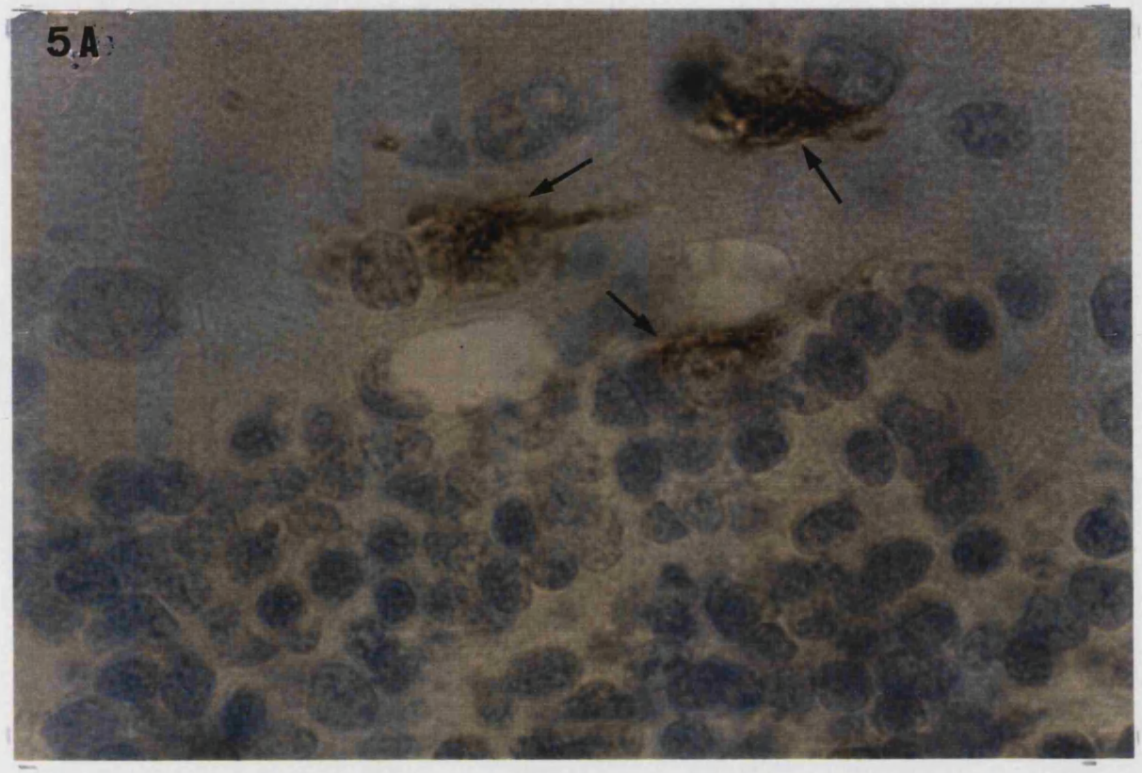
BACKGROUND	REGION OF HYPOTHALAMUS		
	LVR	NLTpp	LVR
	(YOUNGER FISH) (n=1)	(OLDER FISH) (n=5)	
WHITE-REARED	34 ± 3.5	97 ± 12.0	44 ± 2.4
BLACK-REARED	14 ± 1.1**	49 ± 6.6	24 ± 2.7**
W-B	24 ± 2.5	----	----
B-W	42 ± 2.2##	----	----

Table 8.2. 40-60g, single individuals from either white (W) or black (B) tank or two fish transferred from to W to B (W-B) or B to W (B-W) for 6 days, were anaesthetised and the brain perfused with Bouins fixative, and wax-embedded. Sections cut at 8 μm were immunostained with MCH antiserum, and the nuclear area of neurones in the LVR was measured (25 neurones /fish) from camera lucida drawings. Values are μm2.

Five adult fish (200-300g) from both W and B tanks were also processed as above.

** P <0.001 compared to white-reared fish.
P <0.01 compared to W-B and B-reared fish.

Figure 8.5 A and B. Photomicrograph showing the comparison of the nuclear size of the ir-MCH neurones between white **(A)** and black-reared fish **(B)** (arrowed) (See table 8.2 also). Magnification x1000


5A

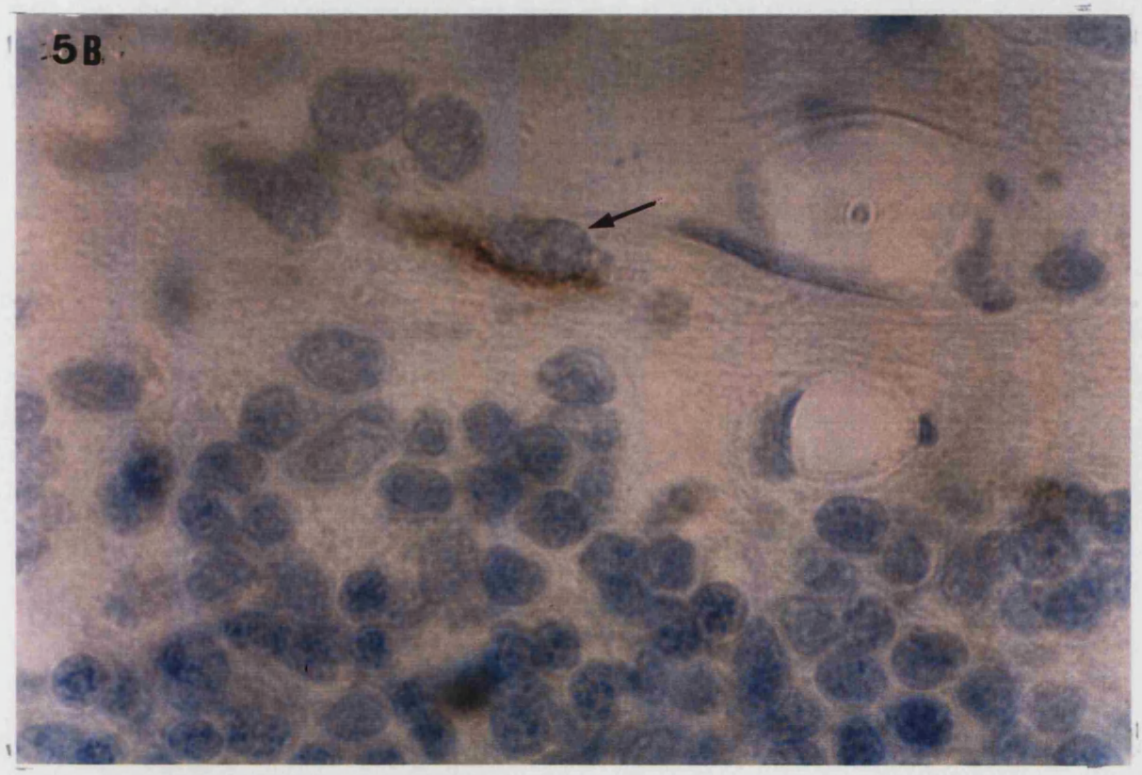

5B

Table 8.3

Influence of rearing conditions on MCH content of the brain and pituitary.

	TISSUE MCH CONTENT (ng)		
BACKGROUND	**PITUITARY**	**NLT**	**LVR & THALAMUS**
WHITE-REARED	1157±110	107±11	7.4±1.7
BLACK-REARED	223±13**	7±0.8**	2.1±0.3**
RATIO W/B	5.2	15.2	3.5

Tissues were extracted in 0.1M Hcl and the MCH content determined by radioimmunoassay. (Data obtained from Suzuki et al., 1995)

** $P<0.001$ compared with white-reared fish.

8.4. DISCUSSION

The present study demonstrates for the first time some properties of a newly described second group of ir-MCH neurones in the hypothalalmus. Cell bodies of this group are closely associated with paraventricular organ, located bilaterally above the lateral ventricular recesses. The neurones located over the LVR often seem to be bipolar when seen in vibratome sections. Their size varies according to their location i.e. smaller immunoreactive MCH cells, tend to be found more medially and closer to the third ventricle of the LVR, while those situated more dorsolaterally tend to be larger. However, they are much smaller than the magnocellular immunoreactive MCH neurones found in NLT. Moreover, results from *in situ* hybridization studies from this laboratory have demonstrated that the overall MCH-2 probe binding associated with this group of cell is much less (approximately 50 folds) than that detected in the NLT (Suzuki et al., 1995).

LVR-MCH neurones project major immunoreactive fibre tracts dorsally into thalamus, coursing on either side of the third ventricle. Such fibres are also seen prominently either side of the posterior commissure and either side of the habenula and in a more ventral, periventricular region at the junction between the thalamus and hypothalalmus (Naito et al., 1985; Bird et al., 1989).

Immunoreactive MCH cell bodies have been demonstrated in the same area in the grass carp and molley (Batten and Baker 1988; Bird et al., 1989). These authors have also found less abundant but distinct ir-MCH fibres within the optic tectum, along the optic

tracts and in other areas. However, it is not yet certain whether the fibres in the various areas arise from the magnocellular NLT-MCH neurones on the floor of the hypothalmus or from the second group of parvocellular neurones above the LVR. This can only be confirmed by the application of retrograde tracers in the fibres and tracing them to their cell bodies.

In addition, vibratome sections showed that short bilateral tracts of fibres were directed ventrally from the LVR-MCH cells towards the ependyma of the ventricular cavity, close to the posterior tubercle (Fig 8.3B). In wax-embedded brains, sectioned at 8 μm, fibres were never seen to project into the ventricular cavity, in the way they do in the lampreys brain (Baker 1988), but frequent examples of MCH contacting blood capillaries were observed (Fig. 8.4B). Whether they do indeed make contact with the ventricular cerebrospinal fluid could be determined by introducing by retrograde tracers such as horseradish-peroxidase (HRP) or cobaltous lysine into the third ventricle.

The different location, size and apparently different projection of fibres into the brain, rather than to pituitary, suggested initially that the LVR-MCH cells might play a completely different role from the MCH cells in the NLT, which obviously produce the neurophypophyseal hormone.

Interestingly, parvocellular neurones, situated above LVR, also showed a strong response to tank colour. although, there is no evidence that their products make a contribution to the hormones in the pituitary gland. This contrasts with the result of a recent study by Groneveld et al., (1995). These authors, reported that these LVR-MCH

neurones were unresponsive to change in background colour. Possible explanation for this discrepancy could be that the LVR-MCH neurones respond to background much more slowly than those in the NLT; the trout in the present study had been maintained all their lives in white or black tanks, whereas the tilapia in Groneveld's study were adapted for only a few weeks. However, a marked change in synthetic activity seemed to be evident within 6 days after transferring trout between white and black tanks suggesting a rapid response. It remains to be investigated whether there is a species difference between teleosts in the conditions that influence the LVR-MCH neurones.

The activity of these neurones expressed either in terms of their numbers or nuclear area, varies both according to the background colouration and the age of the fish (Table 8.2; 3). The abundance of immunoreactive MCH in the NLT, thalamus and pituitary gland of trout also varies according to the age and the colour of the tank in which fish kept (Baker, 1991), as confirmed in the present study.

At present, many questions about the physiological role of these neurones remains to be answered. For instance, the existence of LVR-MCH neurones in the central nervous system of different species of teleosts; the involvement of the LVR neurones in the stress response and the modulation of the activity of the HPI axis. The inhibition of CRF from the hypothalamus and α-MSH from the neural lobe of the pituitary gland by LVR-MCH neurones is other problem which need further scrutiny.

In summary, by using the immunocytochemistry, the present study for the first time identified a new group of MCH in the hypothalamus. Fibres arise from these neurones

are likely to innervate the central nervous system rather than the pituitary gland. Moreover, like the magnocelluar NLT-MCH neurones, the second group of LVR-MCH neurones also seemed to influenced by tank colour.

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